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IMMUNE RESPONSES OF MICE TO PULMONARY
INFECTION WITH BORDETELLA PERTUSSIS

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THE FACULTY OF SCIENCE, UNIVERSITY OF GLASGOW

DEPARTMENT OF MICROBIOLOGY

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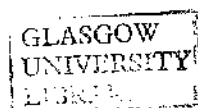
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To Mum, Dad and my husband Allan for their
continual encouragement throughout this work.

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SUMMARY

The object of this investigation was to define the nature of the immune responses of mice that had been given a sublethal pulmonary infection with Bordetella pertussis - an infection which shares many features with whooping cough in man. Both serum antibodies and local, secretory antibodies were studied, the latter being sampled as tracheo-bronchial washings (TBW). A particular focus of interest was in those responses that were directed against biologically active components of B. pertussis and in those responses involved in passive protective activity against intracerebral and sublethal intranasal challenge with live B. pertussis.

Preparatory to this, it was necessary to define the time characteristics of the sublethal pulmonary infection in HAM 1/CR mice given B. pertussis strain 18-323. After instilling a dose of approx 10^5 bacteria via the nose, peak viable counts of 10^7 - 10^8 c.f.u. per lung mass occurred around day 15, after which the count quickly decreased. However B. pertussis was occasionally isolated from mouse lungs as late as 31 or 35 days post-instillation. The peak pathological response occurred around day 18, a few days after the c.f.u. peak. Subsequently there was a plateau where pathological changes varied little.

As a measure of the inflammatory response within the lungs during the sublethal pulmonary infection, lysozyme levels were assayed in pooled TBW and were maximum at day 15 post-instillation. This coincided with the peak colony count in the lungs.

Antibody levels in pooled sera and TBW which were taken from groups of normal and infected mice were assayed in parallel with the U.S. Standard Antipertussis serum. Among the B. pertussis antibodies

serum
in this reference were agglutinins, precipitins, antibodies to filamentous haemagglutinin (F-HA), lipopolysaccharide (LPS) and histamine sensitizing factor (HSF); however no antibodies to heat labile toxin (HLT) were detected. This serum was protective against sublethal intranasal challenge and also, at a lower titre against intracerebral challenge. In both cases, the serum was tested as a mixture with the B. pertussis challenge. Sublethal intranasal passive protective activity was measured by the ability to prevent or reduce both B. pertussis isolation from lungs on lung culture and pathological changes in lungs after infection.

Immune responses to certain components of B. pertussis appeared strongly in serum but were absent from TBW. These serum antibodies included precipitins, agglutinins to B. pertussis X-mode (reciprocal titre of 64) and C-mode (reciprocal titre of 8) and anti-F-HA (reciprocal titre of 32). The agglutinin and anti-F-HA responses peaked respectively day 48 and 34 after infection. Conversely, a strong anti-LPS response (reciprocal titre of 64) was detected in TBW but not in serum despite the fact that serum had a 50-times higher concentration of protein than TBW. No anti-HLT was detected in TBW and sera at any time, but the inherent neutralizability of the toxin preparation was demonstrated with a rabbit hyperimmune serum against HLT-toxoid which served as a positive control. Antibodies to HSF were likewise not detected in mouse convalescent sera or TBW even up to 61 days after challenge (when the longest experiments were ended). However the convalescent mice themselves showed some degree of resistance to histamine-sensitization, compared with age-matched controls, when injected with low doses of pertussis vaccine and then challenged

with histamine. These same mice also showed some degree of resistance to leucocytosis-induction by vaccine. Thus the mice convalescent from the sublethal pulmonary infection had probably developed antibodies to pertussigen.

Pooled normal and acute-phase (15 day) sera contained B. pertussis agglutinins with both having ^{reciprocal} titre of 8, and anti-F-HA with both having ^{reciprocal} titre of 2. The ^{reciprocal} agglutinin titre of individual normal and acute phase sera were investigated and both sera had ^{reciprocal} titres ranging from 2-32. There was no booster effect on ^{the reciprocal} agglutinin titre in acute phase sera which would be expected if the normal agglutinins were due to previous encounters with Bordetella antigens. Individual ^{reciprocal} day 61 mouse convalescent sera had agglutinin titres ranging from 32 to 256.

When given as a mixture with a sublethal pulmonary challenge, pooled convalescent sera and TBW had easily demonstrable sublethal ~~intranasal~~ passive protective activity. The activity appeared on or after 34 days post-instillation and increased 10 fold by day 61 in both sera and TBW to give respective $PD_{50} \text{ ml}^{-1}$ of approx 39,000 and 900. The activity ratio of sera to TBW of 33:1 is slightly less than the protein ratio for these samples (50:1). Neither pooled convalescent sera nor TBW were protective against intracerebral challenge with 100 LD_{50} of B. pertussis - a dose of bacteria approximately equal in c.f.u. to that used for a sublethal intranasal (SLIN) challenge.

Sublethal intranasal passive protective activity was removed from pooled convalescent sera by absorption with Phase I B. pertussis but not Phase IV organisms. Phase I absorption also removed agglutinins. When the pooled convalescent sera or TBW were given

intranasally up to and including 48 hours before challenge, they exhibited passive protective activity. This suggested that a part of the passive protective activity might be due to antibodies of the IgE class.

Further investigation of immunoglobulin (Ig) class(es) responsible for SLIN passive protective activity showed that removal of IgG reduced this activity in pooled convalescent sera and to a greater extent in pooled convalescent TBW. Removal of IgM had no effect. Removal of IgA and IgE also reduced SLIN passive protective activity of pooled convalescent sera and TBW. It therefore appeared that the intranasally passive-protective antibodies in both sera and TBW belonged to the IgG, IgA and IgE classes, with the first mentioned being the most important in TBW.

These results are seen as increasing the value of the murine sublethal pulmonary infection as a model of pertussis in man. They highlight the desirability of examining the respiratory tract and pharyngeal secretions (e.g. nasal washing, saliva) of convalescent humans for the antibody activities detected in mouse tracheobronchial washings.

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preparations used were B. pertussis Phase I 18-323
and Phase IV D30042

LIST OF ABBREVIATIONS

BG	Bordet Gengou Agar
CAA	Casamino acids
cfu	colony forming units
EAE	experimental allergic encephalomyelitis
F-HA	filamentous haemagglutinin
HA	haemagglutinin
HAI	haemagglutination inhibition
HLT	heat labile toxin
HSF	histamine sensitizing factor
IAP	islet activating protein
IC	intracerebral
IN	intranasal
i.p.	intraperitoneal
i.v.	intravenous
LAT	late appearing toxicity
LIN	lethal intranasal
LPF	lymphocytosis promoting factor
LPS	lipopolysaccharide
o.u.	opacity units
PA	protective antigen
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulphate
SLIN	sublethal intranasal

INTRODUCTION

SECTION I BORDETELLA PERTUSSIS

1.1 General introduction

According to Linneman (1979) the first account of whooping cough was given by Guillaume de Baillou in 1640 who reported an epidemic which had occurred in Paris in 1578 and gave details of a patient in a fit of paroxysmal coughing. However the characteristic inspiratory whoop of pertussis was not mentioned.

Bordet and Gengou first examined the organism microscopically in 1900. Later (1906) they cultured it, from the sputum of infected children, on solid medium containing glycerinated-potato extract and 50% (v/v) whole human or rabbit blood. They named it "Le microbe de la coqueluche". MacDonald and MacDonald (1933) confirmed that Bordetella pertussis is the causative agent of whooping cough by inoculating their four children with 140 viable organisms of a culture isolated from a case of the disease. Two of the children were previously immunized and did not develop the disease while the two unprotected children had a full-blown attack of whooping cough.

Marino-Lopez (1952) proposed the generic name Bordetella for the three organisms which had previously been referred to as Haemophilus pertussis, Bacillus parapertussis and Brucella bronchiseptica. This was accepted in the 7th and 8th Edition of Bergey's Manual of Determinative Bacteriology (M. Pittman 1957 and 1974).

Lautrop (1960) proposed the names pertussis and parapertussis for the infections initiated by B. pertussis and B. parapertussis respectively. Parapertussis is a milder disease and tends to have a lower incidence of occurrence. B. bronchiseptica infection has

occasionally been reported in man (MRC Report, 1956; Lautrop, 1960) although it is primarily an infection of animals. Lautrop (1960) proposed that "whooping cough" was the clinical syndrome caused in man by any one of the above three species.

1.2 Morphological and cultural characteristics of *B. pertussis*

B. pertussis is a small Gram -ve coccobacillus, 0.2-0.3 μ m by 0.5-1.0 μ m. Pleomorphism occurs in older cultures and when the organism is undergoing phase variation. The organism is non-motile and non-sporing. Capsules have been reported in young cultures. The organism is aerobic and grows best at 35°C (Pittman, 1970).

Bordet-Gengou (B.G.) and charcoal agar are the main solid media used to grow *B. pertussis* and it is believed that the blood and charcoal in the respective media are required to absorb growth inhibitors such as fatty acids (Pollock, 1947). Despite being a fastidious organism, *B. pertussis* has simple growth requirements. Its source of carbon and nitrogen are L-glutamate, proline or aspartate. Cysteine is the only amino acid which can provide the organism with its organic source of sulphur. However glutathione, a tripeptide, can also do this. Nicotinic acid or nicotinamide fulfill the sole vitamin requirement (Jebb and Tomlinson, 1955).

1.3 Antigenic Variation

A striking feature of *B. pertussis* is its ability to undergo cultural and serological variation. Two main types of variation have been recorded.

(a) Phase variation which occurs after a series of mutations and results in an irreversible loss of a variety of cell properties.

(b) Phenotypic modulations which occur after changes in cultural conditions and are freely reversible.

1.3.1 Phase variation

Leslie and Gardner (1931) found that during repeated subculture 4 distinct serological and cultural forms of B. pertussis could be isolated. They categorised them as Phase I, II, III and IV. Phase I strains were virulent and homogeneous in their agglutination properties, while Phase IV were avirulent, did not agglutinate with Phase I antisera and grew on nutrient agar.

It has since been observed that the loss of virulence and changes in growth requirements happen in a random fashion rather than the stepwise process proposed by Leslie and Gardner (Standfast, 1951; Field and Parker, 1979). Parker has proposed the terms "fresh isolate", "intermediate strain" and "degraded strain" to replace the Phase system of Leslie and Gardner. The biological activities associated with fresh isolates (Phase I in Table 1) are lost in a random fashion and intermediate strains have various combinations of these properties.

1.3.2 Phenotypic variation

There are two main types of phenotypic variation recorded

(i) Antigenic modulation

(ii) Nicotinic acid modulation.

(i) Antigenic modulation

This was first described by Lacey (1951 and 1960) who reported that the various changes in cultural conditions led to phenotypic changes which were due to "reversible and continuously environmentally dependant in metabolism". Lacey (1960) described 3 modes: X, I and C mode. X (xanthic) mode referred to the yellowish ochre hue in the

Table 1 : Comparison of properties of Phase I and Phase IV

B. pertussis

Property	Phase I	Phase IV	Reference
Protective antigen (PA)	+ ^a	-	1, 2, 4
Virulence	+	-	10
Histamine-sensitizing factor (HSF)	+	-	2, 3, 4
Lymphocytosis promoting factor (LPF)	+	-	4
28K, 30K, 88K and 98K envelope polypeptides	+	-	5, 6
Cytochrome d - 629	+	-	6
Adenylate cyclase activity	+	-	7, 8, 9
Agglutinogens	+	-	10
Heat-labile Toxin (HLT)	+	-	4

- | | |
|------------------------------|---------------------------------|
| 1 Kasuga <u>et al</u> (1954) | 6 Dobrogosz <u>et al</u> (1979) |
| 2 Aprile (1972) | 7 Parton and Durham (1978) |
| 3 Kind (1953) | 8 Hewlett <u>et al</u> (1979) |
| 4 Field and Parker (1979) | 9 Endoh <u>et al</u> (1980) |
| 5 Parton and Wardlaw (1975) | 10 Leslie and Gardner (1931) |

a + = present; - = absent

growth of B. pertussis on a B.G. plate; the I-mode is an intermediate mode formed within a narrow range of conditions and C (cyanic) mode described the greyish-blue appearance of B. pertussis grown on B.G. with MgSO_4 added.

The following changes in cultural condition may bring about antigenic modulation:

- (i) ionic composition of the medium, e.g. MgSO_4 in place of NaCl
- (ii) growth at low temperatures, e.g. 25°C in place of 35°C
- (iii) miscellaneous factors such as fatty acids, tellurite and old blood.

C-mode cells lack a variety of factors associated with X-mode cells. These are protective antigen (PA), histamine sensitizing factor (HSF) (Wardlaw et al., 1976); lymphocytosis promoting factor (LPF) (Idigbe, 1979); the adjuvant(s) for reaginic antibody and hyperacute experimental allergic encephalomyelitis (EAE) (Wardlaw et al., 1979); adenylate cyclase (Parton and Durham, 1978); and heat labile toxin (Livey et al., 1978). C-mode cells also lack the 28K, 30K, 88K and 98K bands from the outer membrane (Idigbe, 1979) as determined by SDS polyacrylamide gel electrophoresis (PAGE).

(ii) Nicotinic acid modulation

Culture media used for growth of B. pertussis phase I or X-mode typically contains about $1\ \mu\text{gml}^{-1}$ nicotinic acid. Pusztai and Joó (1967) found that modulation occurred if the concentration of nicotinic acid was increased to $500\ \mu\text{gml}^{-1}$. The change was reversible by further culture in medium containing $1\ \mu\text{gml}^{-1}$ nicotinic acid.

Of the 7 strains tested by these Hungarian workers, all lost

PA and HSF, and 5 did not agglutinate with Phase I specific antisera. None of the strains lost the ability to produce HLT, ... unlike C-mode cells. There is another significant difference between nicotinic acid and antigenic modulation, viz in the former there is a much reduced level of the main agglutinin but with no altered specificity whereas in the latter there is a new C-mode antigen which replaces the X-mode antigen.

Like C-mode cells, nicotinic acid modulated cells lack adenylate cyclase (McPheat, 1980) and the 28K and 30K cell envelope polypeptides (Wardlaw et al., 1976).

1.4 Biologically active components of *B. pertussis*

Many biological activities have been shown to be associated with *B. pertussis*: HLT, endotoxin (LPS), agglutinin, haemagglutinin (HA), adenylate cyclase and the factor designated pertussigen. Munoz and Bergman (1977) proposed the term pertussigen as the single entity responsible for a wide variety of responses which have each been attributed to the following independent factors: PA, HSF, LPP, late appearing toxicity (LAT), Islet activating protein (IAP) and heat labile adjuvant. However the term has not been universally accepted and Pittman (1979) in particular has proposed the term "pertussis toxin".

The various biological activities will now be considered individually.

1.4.1 Heat Labile Toxin

Bordet and Gengou (1909) first extracted heat labile toxin (HLT) from *B. pertussis*. Given intraperitoneally, their preparation killed guinea-pigs and subcutaneously it produced haemorrhagic oedema leading

within 48 hours to necrosis. Wood (1940) found in mice intraperitoneally injected with culture filtrate, small colourless spleens, shrunken livers and peritonitis. Munoz (1963) and Cowell et al (1979) showed the HLT to be located in the cytoplasm.

The toxin's role in the pathogenicity of the disease is uncertain. Standfast (1951) found no relationship between the ability of B. pertussis to infect by the intranasal (IN) or the intracerebral (IC) routes and HLT production. Vaccines which are deficient in HLT are actively protective (Munoz and Bergman, 1977). Antibodies to HLT are not found in human convalescent sera (Evans and Maitland, 1939). However Evans (1944) found that antibodies to a crude preparation of toxin did protect mice against a sublethal challenge when the antiserum was mixed with the bacteria before IN administration. The toxin preparation was crude and therefore the protective antibodies may not necessarily have been directed against HLT. Asada (1953) observed similar pathophysiological changes in guinea pigs given crude HLT as has been observed in the lungs of children who had died from pertussis (Mallory and Horner, 1912).

1.4.2 Lipopolysaccharide

Fisher (1955) proposed that the protective antigen in the mouse lethal intranasal (LIN) test was lipopolysaccharide (LPS), since heating a vaccine at 100°C did not destroy protective ability. Dolby (1965) found that the antigen against which the bactericidal action of serum was directed (in the presence of complement and lysozyme) was LPS. However Ackers and Dolby (1972) noted that the LPS did not induce mouse protective activity against the IC challenge with B. pertussis.

The gross chemical structure of this LPS is similar to that of other gram -ve bacteria except that 2 different polysaccharides and lipids appear to be present (Chaby et al, 1979). B. pertussis LPS possesses all the typical biological activities associated with gram -ve endotoxins (Chaby et al, 1979).

1.4.3 Pertussigen

Highly purified preparations of pertussigen exhibit the following activities: Histamine sensitisation, lymphocytosis promotion, islet activation, heat-labile adjuvancy and mouse-protective activity against an IC infection (Munoz and Bergman, 1977). When purified pertussigen was heated at 80°C for ½ h all these activities were destroyed (Munoz and Bergman, 1977).

Parker and Morse (1973), Krzanowski et al (1976); Ortez (1977) and Lee (1977) all found that pertussigen alters the responsiveness of different cells to adrenergic and other agents, as well as upsetting cAMP metabolism. Recently, Katada and Ui (1982a and b) and Katada et al (1982) on the molecular action of IAP on C6 glioma cells indicated that IAP (pertussigen) causes ADP-ribosylation of a membrane protein, which in turn increases receptor-mediated and GTP-induced activation of adenylated cyclase. This mechanism is similar to cholera toxin although it appears that each causes ADP-ribosylation of different membrane proteins. This work has yet to be confirmed. Wardlaw and Parton (1982) suggested pertussigen interacts with these cell types through sialoprotein receptors.

Most of the published work on this component does not use the name pertussigen but refers to the substance by its pathophysiological activity.

(i) Histamine sensitizing factor

Parfentjev and Goodline (1948) first described histamine sensitivity induced by pertussis vaccine in mice. Fishel et al (1962) reported that β -adrenergic receptor antagonists were capable of producing similar changes induced by pertussis vaccine, ie histamine sensitivity, hypoglycaemia and refractoriness to the hyperglycaemia induced by epinephrine. These authors suggested that pertussis vaccine induces β -adrenoceptor blockade. The basis of this altered responsiveness to epinephrine is not yet understood and results inconsistent with the β -blockade theory (Gulbenkian et al, 1968) have been reported. Lehrer (1979) found that HSF decreased in stability and antigenicity when it was purified. He reported that high doses of HSF sensitized mice within 4-8 h and low doses required 72-96 h. This could not be explained by possible delay in the low doses finding their receptors, since antisera given after HSF could not neutralise the sensitivity.

(ii) Leucocyte promoting factor

Frohlich in 1897 (cited by Morse, 1965) first noted leucocytosis in pertussis cases. Morse (1965) demonstrated B. pertussis vaccine induced in mice a hyperleucocytosis with predominating lymphocytosis. This appears to be due to redistribution of cells from the lymphatic tissue to the circulation. The lymphocytes seem unable to migrate out of the circulation at the appropriate site. It is not known if this is due to some alteration of the capillaries or the cell surface of the lymphocytes. Idigbe (1979) found the responsiveness of mice to the lymphocytosis effect of pertussis vaccine was 4-fold increased if the vaccine was administered intravenously instead of intraperitoneally.

(iii) Lymphocytosis promoting factor haemagglutinin (LPF-HA)

Arai and Sato (1976) first isolated this spherical (6 nm in diameter) component and found it also had relatively low haemagglutinating activity. The haemagglutinating activity is sensitive to sialoproteins (Irons and MacLennan, 1979). It was first found to develop low IC protection (Morse and Morse, 1976; Arai and Sato, 1976; Irons and MacLennan, 1979). However it has since been found to produce active protection by this route (Munoz and Bergman, 1977 and 1979). Antisera to this protein protects against sublethal intranasal (SLIN) infection (Sato et al, 1981).

(iv) Islet activating protein

Sumi and Ui (1975) found pertussis vaccine given by the intra-peritoneal route to rats induced an enhanced hyperinsulinaemia response to insulin secretagogues. Whereas epinephrine in normal rats inhibits glucose induced hyperinsulinaemia, this is reversed in pertussis sensitized rats and a hyperinsulinaemia response occurred. Islet activating protein (IAP) was then isolated from B. pertussis culture supernate (Yajima et al, 1978) and shown to produce the same changes in both the intact rat and the perfused pancreas as whole-cell vaccine.

1.4.4 Agglutinogens

Bordet and Sleeswyk (1910) demonstrated that B. pertussis stimulated agglutinins in animals. Much later Andersen (1953a) showed that strains were not homogeneous in their serotype. Eldering et al (1957) designated the three most common agglutinogens as 1, 2

and 3 and since then eight agglutinogens have been demonstrated. Agglutinin 1 is common to all strains. Spheroplasts free of agglutinin 1-6 produce an effective vaccine against IC challenge (Ross and Munoz, 1971; Dolby and Brome Shanbury, 1975). However Preston (1966) has criticised the mouse protection test as a suitable measure of vaccine potency and proposed the agglutinogens as protective antigens in man.

1.4.5 Filamentous haemagglutinin

Keogh et al (1947) first isolated haemagglutinin (HA) from pertussis and suggested it as a protective antigen in experimental murine infections. Masry's (1952) preparations of HA were antigenic but did not protect mice against IN or IC infection. Sato et al (1974) isolated a purified HA which protected mice against challenge and also promoted lymphocytosis. On further purification two distinct haemagglutinins were isolated with different properties, filamentous haemagglutinin (F-HA) and LFF-HA (Arai and Sato, 1976; Morse and Morse, 1976)

According to Arai and Sato (1976) the former component is a protein, has high haemagglutinating activity, consists of 2 x 40 nm filaments of molecular weight 130,000. Irons and MacLennan (1979) found its activity to be sensitive to cholesterol. It possesses no lymphocytosis promoting activity. Arai and Sato (1976), Morse and Morse (1976) and Irons and MacLennan (1979) found it developed active protection against the IC infection. Antiserum to this F-HA also passively protects against a SLIN infection (Sato et al, 1981). However on further purification this component was protective actively and passively only against a SLIN infection (U.S. Workshop). Sato

et al (1979) reported the interaction of specific anti F-HA with pili-like structures of B. pertussis. It was widely believed that F-HA was derived from pili and often the term fimbrial haemagglutinin has been used. However, Ashworth et al (1982) recently indicated that pili did not label with anti F-HA, but instead with antibody towards a serotype-specific agglutinin 2.

1.4.6 Adenylate cyclase

Phase I B. pertussis have high levels of extracytoplasmic adenylate cyclase, a location peculiar to pertussis and not shown by mammalian or other microbial cells (Hewlett et al (1979). Phase IV and C-mode B. pertussis have negligible levels of the enzyme. Wardlaw and Parton (1979) proposed that the enzyme was involved in the expression of biological properties known to Phase I organisms but not present in Phase IV or C-mode.

1.4.7 Others

A number of other active substances of B. pertussis have been described which are less well characterised. These include a haemolysin, a neurotoxin, a polymorphonuclear, leucocyte-inhibiting factor, a factor which affects macrophage function (Wardlaw and Parton, 1983) and a tracheal cytotoxin (Goldman et al, 1982). This tracheal cytotoxin recently described by Goldman et al (1982) which causes ciliostasis in vitro may also have a prime role in lodgment.

SECTION II B. PERTUSSIS INFECTIONS OF MAN

2.1 Pertussis - The disease

Pertussis is an acute localized disease of the respiratory tract for which man is the only natural host (Pittman, 1970). Sub-clinical or clinically unrecognized infections do occur but there is no evidence that chronic carriers are significant in the transmission of infection. The disease is spread primarily by droplets expelled from the respiratory tract (Linneman, 1979). Pertussis is highly communicable. The disease has four distinct stages and the average length of the illness is around 60-70 days (Pittman, 1970).

The first stage is the incubation period which lasts between 7-14 days (Court, 1953), its length varying with the infecting dose (Olson, 1975). The onset of symptoms begins in the catarrhal stage with sneezing, anorexia, a little fever and ends with an increasingly severe cough. This stage also lasts between 7-14 days (Court ^{et al}, 1953). The disease is highly contagious at this stage with attack rates of 80-90% in unvaccinated children exposed at home (MRC, 1951). The paroxysmal stage follows and persists for 2-6 weeks or longer; fever is usually absent from this stage (Dayal et al, 1969). The severe bouts of coughing or paroxysms, which occur without warning during this stage, may be as frequent as 10-21 per day and may be accompanied by the classical whoop which is caused by a long inspiration of air. Vomiting may also follow these paroxysms (Linneman, 1979) and lymphocytosis and hypoglycaemia are usually present at this stage. Most of the complications of pertussis are by products of the paroxysmal coughing and its associated lung damage and cerebral anoxia. Death

may occur at this stage (Olson, 1975).

The symptoms associated with the paroxysmal stage begin to subside gradually as the disease enters the convalescent stage. However it may take up to 6 months for paroxysms to cease (Olson, 1975). Pulmonary and neurological sequelae may be present as a result of the disease. Pulmonary sequelae have a low frequency and take the form of bronchitis and pneumonia (White, Finberg and Tramer, 1964). Neurological sequelae have a much higher incidence (Olson, 1975). In a follow-up report of 35 children over a 14 year period 9 children showed evidence of more prolonged intellectual and emotional difficulties (Byers and Rizzo, 1950). The remaining 26 apparently recovered without suffering any ill effects. Six of the nine children had permanent handicaps with 4 showing encephalopathy.

Farber and Vawter (1961) and Olson (1975) have reported adenovirus isolation from clinically diagnosed cases of whooping cough. These viruses may cause paroxysmal coughing but not the inspiratory whoop or lymphocytosis which characterise the pertussis syndrome (Linneman, 1979).

Pertussis is found worldwide but has a higher incidence in countries with lower socio-economic conditions such as overcrowding (World Health Statistics Report, 1977). Epidemics of pertussis occur in cycles of 3-4 years (J.C.V.I., 1977).

2.2 Laboratory findings

2.2.1 Pathology

Autopsy of a child who had died of pertussis showed organisms on and between the cilia of the epithelium on the tracheobronchial

tree (Mallory and Hornor, 1912). Infected epithelial cells had been shed and cilia reduced to stubs. Organisms had not penetrated into the epithelial cells. Polymorphs, lymphocytes and plasma cells were all present and bacteria were observed within the phagocytic cell. Olson (1975) suggested that the organism would find amino acids as its carbon source in the mucus of these regions.

Collier et al (1977) showed that tracheal organ cultures with ciliated epithelial cells attracted B. pertussis which attached to the cilia and microvilli. The bacteria did not attach to non-ciliated cells. Ciliostasis followed after attachment. Standfast (1958) suggested that heat labile toxin paralysed the cilia and thus prevented the organism from being washed away by mucus. Matsuyama (1977) found Phase I but not Phase III attached to the lung but both caused ciliostasis suggesting attachment is the important step in establishing infection. Sato et al (1979) proposed that pili attach the organism to the ciliated epithelium but direct evidence of this was not forthcoming (Matsuyama, 1977).

Although B. pertussis remains localized in the lung, pathological changes have also been observed in the central nervous system (Dolgopol, 1941). Oedema and haemorrhages were present and were consistent with anoxia and venous pressure.

2.2.2 Bacteriology

B. pertussis is rarely isolated from the host after the second week of the paroxysmal stage (Linneman, 1979). The isolation rate after the third week of infection is around 50% (Brooks et al, 1942). Collection of specimens by the pernasal swab method (Cruikshank, 1944) and direct plating of specimens onto B.G. medium gives the best chance

of recovery (Parker and Linneman, 1980). Rapid diagnosis by fluorescent antibody technique has the disadvantage of giving false positives (Chalvardjian, 1966).. Treatment of a case with antibiotics decreases the chance of a positive culture but has little effect on the clinical symptoms unless started within 8 days of symptom appearing (MRC Report, 1953). Bass et al (1969) found erythromycin was the most effective, when administered before the paroxysmal stage, at reducing the length and severity of the illness. Hyperimmune human serum like antibiotic therapy, is said to be effective in eradicating organisms from the host but ineffective in reducing the severity of infection unless administered before the onset of the paroxysmal stage (Bass et al, 1969). However there has been no controlled trial of pertussis immune serum.

2.2.3 White blood cell count

Kaufman and Bruyn (1960) found 57% of hospitalized patients had a white blood cell count greater than $20,000 \text{ mm}^{-3}$ with admissions showing a range between $8,000 \text{ mm}^{-3}$ to $140,000 \text{ mm}^{-3}$. They found the average count in complicated cases to be greater than that of uncomplicated cases. However the variability was so great that complications could not be predicted by this means. Lagergren (1963) found leucocytosis less frequent in patients below 6 months of age.

In the cases showing leucocytosis, 63% of these exhibited lymphocytosis (Lagergren, 1963). Lymphocytes comprise 50-75% of the total white cell count (Kaufman and Bruyn, 1960). The cells are for the most part normal-appearing mature, small T and B lymphocytes in equal proportions (Tsukimoto and Lampkin, 1976). Treatment of patients

with antibiotics produced a rapid decline in white blood cell count within 24 hours (Kaufman and Bruyn, 1960).

Eosinophilia was observed by Biro (1960), Klimt (1961) and Olson et al (1964). The latter found this phenomenon to be more pronounced in convalescence and defined the syndrome as acute infectious lymphocytosis. However Reed et al (1970) were unable to confirm it.

2.2.4 Biochemistry and pharmacology

Regan and Tolstouhiov (1936) described the disturbances induced by whooping cough in blood chemistry. Inorganic phosphorus and pH were diminished usually by the end of the catarrhal stage and significantly diminished during the first weeks of paroxysms. Carbon dioxide combining power remained normal but due to inadequate respiration particularly after the onset of paroxysms CO_2 accumulated in the blood to cause an increased concentration of carbonic acid. Calcium concentration shows slight instability. Blood sugar was at the lower limits of the normal range and became hypoglycaemic at the end of the paroxysmal stage. This drop in blood glucose could not be explained by reduction in food intake since the children ate normally. Uric acid was present at increased levels.

Other metabolic disturbances have been observed. Badr-El Din et al (1976) found that attenuation of epinephrine induced hyperglycaemia persisted for at least 50 days in children with whooping cough. Sanyal (1960) examined 30 children in the typical stage of whoop in the age group 1-10 years, for sensitivity existing to histamine, 5-hydroxytryptamine and 48/80, a histamine liberator. It was shown that nearly

all were maximally sensitive to histamine and slightly sensitive to 5-hydroxytryptamine as measured by the area of erythema around the lint soaked in an aqueous solution of the testing substance. The 48/80 did not cause any reaction.

2.3 Resistance to infection

2.3.1 Natural immunity

There appears to be no passive transfer of protective anti B. pertussis antibodies from mother to foetus (Lorber, 1975; Preston et al., 1974). The infant below one year of age and especially below 6 months of age, is very susceptible. Despite this, it may not be practicable to start immunisation much before 3 months of age because of poor immune response (J.C.V.I., 1981).

In regard to duration of immunity after infection, Mannerstedt (1934) found 29 cases of adult infections and three infections in children between the ages of five and 15 years all of whom had previously had whooping cough. However second attacks are rare and are usually not diagnosed (Pittman, 1970).

The nature of the immunity that develops after infection has been little studied. Pittman (1979) proposed that local secretory immune responses gave a short-term immunity by preventing attachment of the organisms to the ciliated epithelium. More durable immunity, lasting for a few years, might be due to serum antibodies against pertussis toxin (pertussigen).

2.3.2 Vaccination

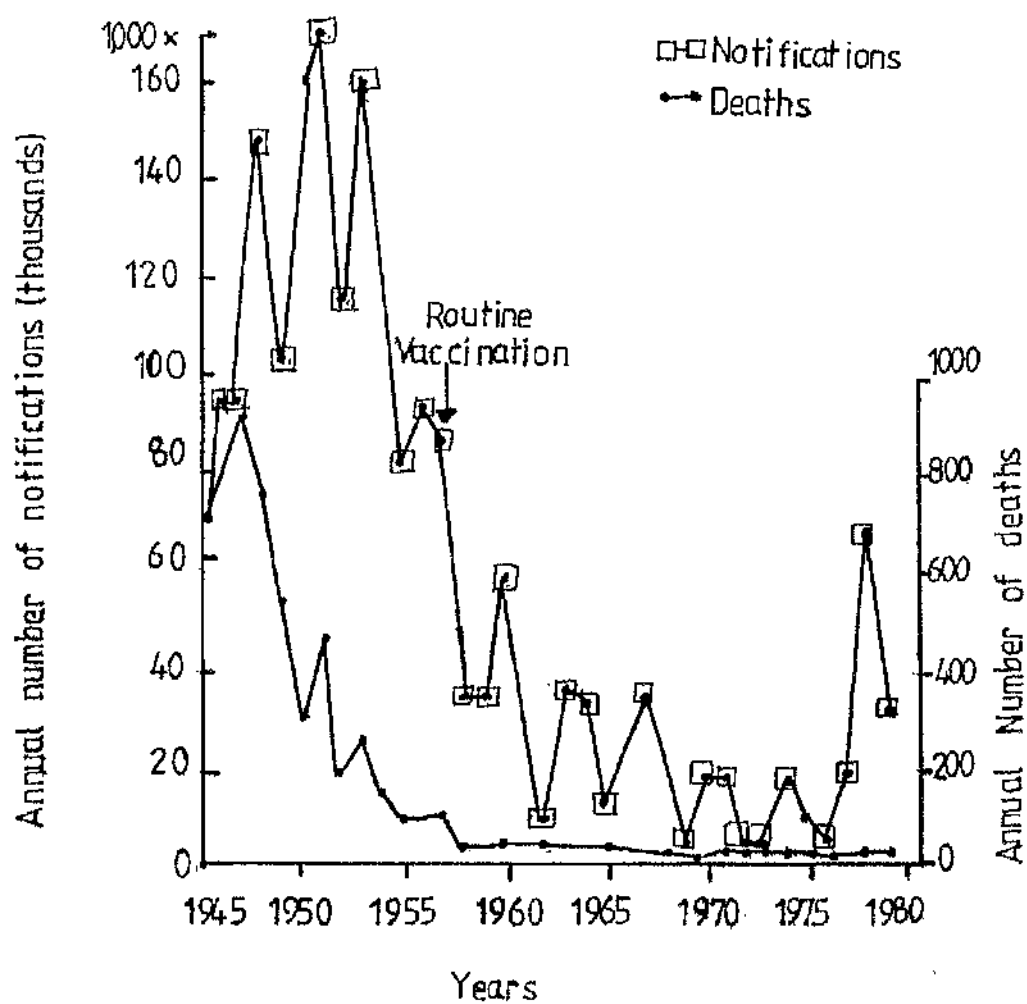
Madsen (1925, 1933) studied pertussis vaccine efficacy during

two epidemics in the Faroe Islands. He found vaccination significantly reduced the severity of the disease and if completed before the onset of an epidemic, the rate of infection was reduced from 98% to 75%. More encouraging results were found in subsequent vaccine trials. In these the criteria for cultural conditions which are required to give Phase I virulent organisms (Leslie and Gardner, 1931) were used for vaccine production. Kendrick and Eldering (1939), in a controlled study, found 35% vaccinated children exposed to infection in the home contracted pertussis compared with 89% of unvaccinated children.

The initial controlled studies of the Medical Research Council (1951) revealed a reduction in home exposure attack rate (H.E.A.R.) averaging around 70% in vaccinated children and also a reduction in the severity of illness. Many vaccines were tested in this and the subsequent MRC field trials reported in 1956 and 1959. Not all vaccines produced a worthwhile reduction in the H.E.A.R. These later two trials established an inverse correlation between H.E.A.R. and vaccine potency in the intracerebral mouse protection test (Kendrick et al, 1947). The World Health Organisation (WHO) subsequently recommended this test as the standard assay of pertussis vaccine protective potency (W.H.O. 1964; 197). Pertussis vaccine, combined with diphtheria and later also with tetanus toxoids, was introduced in the United Kingdom on a national scale in 1957 (Figure 1).

In a report by the Public Health Laboratory Service Working Party (1969) on the efficacy of whooping-cough vaccines, it was subsequently shown that vaccination at that time was not significantly reducing the attack rate. Preston (1963) had already shown a shift in the serotype of organisms causing infections, ie from serotype 1,

Figure 1. Whooping cough in England and Wales from 1945-1980



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2, 3 to serotype 1, 3. Perkins (1969) found British vaccines contained only one-half the international standard dose of 4 I.U. A mineral adjuvant, aluminium hydroxide, was incorporated as this was found to provoke a greater immune response than the plain preparations previously in use as well as reducing the toxicity of the vaccine (Burland et al, 1968). A major consideration in the routine vaccination of children is that it induces herd immunity which in turn minimises the risk of infection in the very young infant not yet immunized (Stuart-Harris, 1979).

Adverse reactions to pertussis vaccines have been well documented. The earlier worker (Madsen, 1933) recorded two deaths after pertussis vaccine. Byers and Moll (1948) found in 15 vaccine associated reaction cases admitted to hospital, all but one showed evidence of impairment to the nervous system. An examination of the family histories showed a high incidence of neurological abnormalities.

In the mid-70's there was much publicity to the adverse neurological reactions to pertussis vaccine by the Association of Parents of Vaccine-Damaged Children and by Kulenkampff et al (1974). This led to a decline in acceptance rate in the United Kingdom by about 40% (Stuart-Harris, 1979).

Subsequently a study by the Joint Committee on Vaccination and Immunization on adverse reaction in hospitalised children under 3 years of age was published in 1981. It concluded that permanent damage as a result of pertussis immunization was a rare event - around 1 in 310,000 immunizations, and that attribution of a cause in individual cases was precarious.

2.3.3 Passive immunization

Debre (1923) found that serum from patients who had been infected for four weeks had some immediate protective ability for intimately-exposed children to whom it was given during the incubation period of pertussis. McGuinness et al (1944) reviewed the use of human hyperimmune serum. They reported that it dramatically reduced the mortality rate if given in adequate dosage compared to patients given no sera. There was also therapeutic value for exposed children but here the dose was increased.

Anes et al (1953), Kaufman and Bruyn (1954) and Bass et al (1969) found hyperimmune globulin treatment did not alter the course of the disease unless treatment began before the catarrhal stage compared to patients given no sera. Only in a few cases was the length of the paroxysmal stage reduced.

2.4 Nature of the immune response after infection

Many parameters of the immune response after a B. pertussis infection have been investigated with a view to relating severity of disease to one or more immunological tests.

Kendrick et al (1937) found blood from B. pertussis infected children had an increased degree of phagocytosis as well as an increased number of phagocytic cells taking part. This response was B. pertussis specific. This was demonstrated by incubation of blood with 20×10^9 B. pertussis ml⁻¹. A drop, 5 mm in diameter, of antigen-blood mixture was placed on a slide, dried and stained. Twenty five polymorphonuclear cells were examined and B. pertussis contained within were counted.

The polymorphonuclear cells of blood from uninfected children contained no or very few B. pertussis organisms, whereas in infected children's sera increasing numbers of polymorphs contained many organisms as the disease progressed. The maximum opsonocytophagic response occurred at eight weeks after the onset of infection and remained elevated for the next four months. However there was no definite relationship between opsonocytophagic response and the severity of the disease in the 28 children examined.

Winter (1953) examined the development of antibacterial agglutinins, antihaemagglutinins and protective antibodies as measured by the LIN mouse protection test. 62% of patients developed B. pertussis agglutinins but only 14% developed antihaemagglutinins. Sera tested during the acute stage of the infection had either no protective activity or a $PD_{50} \text{ ml}^{-1}$ between 5-10 against a LIN murine infection. However during convalescence the $PD_{50} \text{ ml}^{-1}$ showed a 4 to 10-fold increase to 11-30. The maximum titre occurred between the 5th to 8th week of disease, protective antibody was present in all sera tested and its development paralleled the course of recovery.

Dolby and Stephens (1973) were unable to relate the immunity of the infected child to titres of either agglutinin or bactericidal antibody. The proportion of children with both these antibodies were higher in bloods taken between 2-6 months after appearance of symptoms than in bloods taken between 1-2 months. Older children of 1 to 6 years who did develop agglutinins did so within 1 month of infection, whereas infants below 1 year of age produced agglutinin only after months. However the frequent absence of both bactericidal and agglutinin antibody response was noted. All sera examined had antihaemagglutinins in bloods taken after 2 weeks to 4½ months from

onset of symptoms. The protective ability of sera of infected children bled 1-12 weeks after infection measured in mice against a small lethal brain infection was present in 66% of the cases.

The distribution of agglutinins, bactericidal antibodies and antihaemagglutinins in serum IgG and IgM was examined. Infants below one year of age had a higher antihaemagglutinin titre in the 19S globulins than 7S globulins. In older children the titre was similar for both 7S and 19S. Levels of agglutinins and bactericidal titres in both 7S and 19S was similar in children of all ages.

Bradstreet et al (1972) proposed that the complement fixation and immunofluorescence tests were the best routine serological tests for pertussis, particularly in the 6 months or over age group. Ninety-five percent of patients had raised antibody titre in paired sera during infection.

Aftandelians and Connor (1973) using the Ouchterlony technique to examine the development of precipitins, found that such antibodies to sonicated B. pertussis developed in 86% of paired sera from pertussis cases. In contrast agglutinins developed in only 42% of paired sera.

2.5 Nature of the immune response after vaccination

Dolby and Stephens (1973) were unable to relate the immunity of the child to the titre of the agglutinin, bactericidal antibody or protective ability of sera against a small, lethal brain infection. Aftandelians and Connor (1973) found precipitin lines to B. pertussis sonicate by the Ouchterlony technique in only 36% of samples from vaccinated children. Bradstreet et al (1972) found complement fixing antibody undetectable 7 months after the last injection of the

vaccination programme. Macaulay (1979) found indirect haemagglutination, complement fixation and agglutination antibody response developed, although not always together, in pertussis vaccinated children. Indirect haemagglutination developed more frequently than the other two antibodies in paired sera. IgG was the only Ig class involved in complement fixation test whereas IgG and IgM were involved in indirect haemagglutination (Macaulay, 1981).

SECTION III EXPERIMENTAL MODELS OF B. PERTUSSIS OTHER THAN THE MOUSE

3.1 Animal models

Various experimental animal models other than the mouse have been studied for their ability to reproduce the clinical changes and laboratory findings associated with the pertussis syndrome. Pertussis infections in primates have been most successful (Table 2).

The B. pertussis strain and infecting dose are rarely mentioned in the early literature on primate infections with B. pertussis. However Huang et al (1962) infected the Taiwan monkey with 20×10^9 organisms of the 18-323 strain and Stanbridge and Preston (1974) infected the marmoset with 50×10^9 organisms strain 41633.

Comparative efficacy of the various methods of administering B. pertussis infection to the respiratory tract has not been established and intratracheal, IN and spraying into the nose have all been used as a mode of infection. However the two most thorough investigations by Lin (1958) and Huang et al (1962) both infected the monkey by spraying the nose. The number of animals used has generally been small (3-11) except with the experimental infection of the Taiwan

Table 2 : Primate animal models of Pertussis

Primate	Strain and infecting (dose of <u>B. pertussis</u>)	Method of administration and No. of animals infected	Clinical observations	Laboratory findings	Reference
Macacus	Not mentioned	Intratracheally 3	None	Not investigated	Mallory <u>et al.</u> , (1933)
Macacus	Fresh isolates	Swab pharynx 1	Paroxysmal cough within 10 days. Vomiting.	None	Inaba (1912)
Macacus and Cebus	Fresh isolates 1-2 cc (2-4 billion organisms)	Swab pharynx and/or injected into larynx 8	Paroxysmal cough after 1-3 weeks, incubation period 10-21 days. 3 deaths.	<u>B. pertussis</u> on respiratory epithelium, leucocytosis (14,000-38,000 mm^3) lymphocytosis at same time as paroxysmal cough. Recovered animals immune	Sauer and Hambrecht (1929)
Erythrocebus	Not Mentioned	Intratracheally 11	Interstitial mononuclear pneumonia in 8 out of 9. 2 deaths.	Lymphocytosis in 6 out of 9. Multiplication of organism absent.	Sprunt <u>et al.</u> , (1938)

Table 2 (contd.)

Primate	Strain and infecting dose of <u>B. pertussis</u>	Method of administration and No. of animals infected	Clinical observations	Laboratory findings	Reference
<u>Macacus cyclopsis</u>	18-323 F2 10-5362 23-757	Spray through nose into throat 10	Paroxysmal cough started 2-12 days after infection. Started lasting 3-14 days with or without whoop. Immune to re-infection.	Isolation of <u>B. pertussis</u> until 5 weeks into infection. Opsonocytophagic index increased leucocytosis (13,000-34,000 mm^{-3}) and lymphocytosis rapid agglutination titre increased only after reinfection.	Lin (1958)
Taiwan monkey	18-323 (20×10^9)	Spraying into nares and pharynx 42	Incubation period 7-15 days and paroxysmal cough lasting 24-64 days. Temperature normal and no loss of weight. Active immunization by vaccine. Passive immunization by vaccine.	Lymphocytosis (15,000-42,600 mm^{-3}). Positive culture until 36 days after spraying. Positive agglutination reciprocal titre 32 in 8 out of 9 sera.	Huang et al (1962)
Marmosets and Rhesus monkey	41633 (50×10^9)	Pernasal "	Colonisation, catarrhal stage. None	None None	Stanbridge and Preston (1974)

monkey where 42 were used.

Clinical observations of pertussis infections in some of the primates indicated a disease pattern closely similar to that in man. Interstitial mononuclear pneumonia was found in 8 out of 9 *Erythrocebus* monkeys (Sprunt et al, 1938). Huang et al (1962) found that the temperature of Taiwan monkeys was normal throughout infection and no weight loss occurred. These animals developed a paroxysmal cough.

The paroxysmal cough has also been recorded in other species of monkey (Lin, 1958). However, Mallory et al and Stanbridge and Preston failed to find it in the *Macacus* monkey or marmoset respectively. The length of time before paroxysmal coughing begins after infection ranges from two days to 3 weeks incubation. Inaba (1912) recorded vomiting after paroxysms.

The induction of immunity to reinfection in the monkey has been demonstrated (Sauer and Hambrecht, 1929; Lin, 1958). Active immunization by vaccination was possible in the Taiwan monkey (Huang et al, 1962). Passive immunization of this species was also successful with sera from convalescent and vaccinated Taiwan monkeys (Huang et al, 1962).

Laboratory findings in pertussis-infected primates are also similar to pertussis in man. Actual pulmonary multiplication of the bacteria has not been demonstrated, but colonization persisted until the 46th day (Lin, 1958; Stanbridge and Preston, 1974). Leucocytosis ($13,000-38,000 \text{ mm}^{-3}$) with predominately lymphocytosis has been demonstrated (Sauer and Hambrecht, 1929; Lin, 1958). Lymphocytosis alone has also been observed (Sprunt et al, 1938; Huang et al, 1962). The opsonocytaphagic index in infected *Macacus cyclopsis* increased

Table 3 : Rodent and Carnivore animal models of pertussis

Species	Strain and dose of <u>B. pertussis</u>	Method of administration and No. of animals infected	Clinical observations	Laboratory findings	Reference
Rabbit	N.M. ^a	Intratracheal N.M.	None	None	Klinenko (1908)
Rabbit	N.M.	Intratracheal 5	Emaciation	Histological lesions in the ciliated epithelium of the bronchi and bronchioles	Mallory, Hornor and Henderson (1913)
Rabbit	N.M.	Intratracheal 17	N.M.	Interstitial mononuclear pneumonia. Lymphocytosis within 2-4 days of infection	Sprunt, Martin and McDearman (1938)
Rabbit	353/Z 18-323 360E 41633 (50 x 10 ⁹)	pernasal N.M.	Catarrh No coughing or vomiting. Active immunization with correct agglutino-gen range prevented infection.	Agglutination development throughout infection.	Preston, Timewell and Carter (1980)
Rabbit	M2 (10 ⁶)	IN	Colonisation asymptomatic	None	Ashworth, Fitz-george, Irons, Morgan and Robinson (1982)

Table 3 (contd.)

Species	Strain and (infecting dose of <u>B. pertussis</u>)	Method of administration and No. of animals infected	Clinical observations	Laboratory findings	Reference
Guinea pig	N.M.	Intratracheal N.M.	None	None	Klimenko (1908)
Rat	N.M. (8.5×10^6)	Intranasal N.M.	Cough like paroxysms	Non-specific inter- stitial pneumonia	Hornibrook and Ashburn (1939)
Dog	N.M.	Intratracheal 96	Infection of normal puppies by intimate contact. Death within 2-6 weeks. Sneezing and spasmodic coughing.	None	Klimenko (1908)
Dog	N.M.	Intratracheal N.M.	Infection of normal puppies by intimate contact. Characteristic lesions. Spasmodic coughing.	Reisolation of organisms between 15-25 days after infection.	Mallory, Hornor and Henderson (1913)

a NM = Not mentioned.

(Lin, 1958). The agglutination titre was either low with a reciprocal agglutination titre 32 (Huang et al, 1962) or only present after reinfection (Lin, 1958).

A range of rodents and carnivores have also been studied. Again only the more recent papers state the infecting dose (Hornibrook and Ashburn, 1939; Preston, 1980). The method of administration was either IN or intratracheal.

Klimenko (1908) was unable to infect rabbits whereas Sprunt et al (1938) and Preston (1980) were both successful in establishing infection in this species. Klimenko (1908) observed infection of normal puppies by intimate contact with sneezing and coughing puppies (following infection). This result was confirmed by Mallory et al (1913) who were also able to reisolate B. pertussis between 15 to 25 days after infection. These latter authors also infected rabbits and observed emaciation after a few days and histological lesions characteristic of pertussis on the tracheobronchial tree. Mallory et al (1913) later acknowledged that their clinical and laboratory finding could have been due to B. bronchiseptica.

^{et al}, Preston (1980) observed that colonization of the rabbit nasopharynx persisted for as long as nine to ten months. The serotype of the infecting strain changed with time. Catarrh was present but no coughing or sneezing was reported. Active immunization was effective only if a range of serotypes was present in the vaccine. Hornibrook and Ashburn (1939) found cough-like paroxysms and non-specific interstitial bronchopneumonia in rats infected with B. pertussis.

Active immunization of rabbits with either outer membrane proteins, F-MA or standard vaccine induced enhanced clearance of B. pertussis. However there was no correlation between elimination

of B. pertussis and serum antibodies to outer membrane proteins, F-HA, LPS, LPF or agglutinin 3. However nasal IgA to F-HA inversely correlated with bacterial persistence (Ashworth et al, 1982).

Multiplication of B. pertussis in the lung has not been demonstrated in any of the animal models.

3.2 Other models

Chick embryos are susceptible to infection with B. pertussis by inoculation of the amniotic fluid (Gallavan and Goodpasture, 1937). Infection occurred on the ciliated bronchi and bronchiole epithelium in preference to the trachea and also on the ciliated oesophageal epithelium. B. pertussis produced reproducible cell damage in cultures of human and cat brain tissue (Felton et al, 1954). The introduction of specific antiserum appeared to halt the process of damage produced by bacteria where normal serum did not.

Organ culture of Phase I, C-mode and Phase IV B. pertussis on hamster trachea has been done by Muse et al, (1977). C-mode and Phase IV organisms were unable to produce ciliostasis over a 48 h period, whereas ciliary paralysis had taken place by this time with phase I organisms.

SECTION IV B. PERTUSSIS INFECTION OF MICE

There are two sites in which B. pertussis organisms multiply in mice: the lungs (Burnet and Timmins, 1937) and the brain (Kendrick et al, 1947). Both are localized infections of ciliated epithelium. Proom (1947) found that B. pertussis did not multiply in the peritoneal

cavity or the bloodstream after an i.p. injection of the living bacteria. Pertussis in man is a localized infection of the upper respiratory tract and the IC infection with these bacteria does not occur in the human species. However in the brain of the mouse, B. pertussis is isolated from the normal protecting mechanism and the bacteria survive and multiply.

4.1 Intracerebral infection

4.1.1 Histological changes

Berenbaum et al (1960) investigated two different routes of IC infection with strain 18-323, (a) through the foramen magnum and (b) through the parietal bone, the route used by Kendrick et al (1947). No difference was found between the two routes except where the lesions were made by the needle.

Multiplication of the bacteria was confined to the ciliated epithelium and ependyma and although some organisms entered the bloodstream, they did not multiply out of the cranial cavity. No organisms were detected in the lung, spleen and liver throughout the course of this infection.

The polymorph reaction which was visible after 24 h in the meninges and choroid plexus had by day 3 moved into the ventricles. Organisms were present in clusters in the supra-optic recess of the third ventricle and occasionally on the lateral ventricular choroid plexus. Extensive areas of haemorrhages were present by day 5 and by day 6 purulent fluid distended the ventricles and extensive polymorph invasion was evident. Organisms either free or within

polymorphs were now more apparent in the ventricles than in the meninges or choroid plexus. Death followed.

Liver, spleen and adrenal glands had some histological changes whereas the lungs, heart, kidneys and pancreas did not. The liver had a terminal fatty change and atrophy of the lymphoid follicles of the spleen occurred. The adrenal glands changed markedly. Lipid depletion began on Day 5 leaving only small areas of lipid in the zone glomerula and innermost cortex. Enzymatic activity of succinic dehydrogenase, non-specific esterase and acid phosphatase in the adrenal cortex increased.

4.1.2 Factors influencing virulence

Standfast (1958) found the LD₅₀ of the IC infection of strain 18-323 remained constant over a mouse weight range of 12-36 grams in an experiment where weight was an indicator of age. He examined the effect of subculture on the virulence of 5 strains. All maintained the same virulence by the IC route after 267 subcultures. However this virulence was eventually lost, although the number of subcultures was not stated. Rough or Phase IV organisms were avirulent. Standfast also found virulence was not influenced by age of culture over a 24 hour to 72 hour range.

4.1.3 Multiplication rate

Berenbaum et al (1960) using strain 18-323 found an initial lag phase in the brain lasting 24 h but by 48 h the organisms were rapidly multiplying. Growth tended to level off about Day 5 when approx 10^8 to 10^9 organisms were present in the brain before death.

Standfast and Dolby (1961) examined growth curves of a range of infecting doses. As the infecting dose was lowered from 10^5 to 10^1 viable organisms, the critical level at which death occurred also decreased from $10^{8.5}$ to $10^{7.8}$ organisms respectively. As the infecting dose decreased the time taken to reach this critical level increased from 4 to 13 days respectively.

High virulence strains such as 18-323 (Berenbaum et al, 1960; Dolby and Standfast, 1961) and 2-Atox (Andersen and Bentzon, 1958) produce a lethal infection (Adams, 1970). Active multiplication continues even after the blood brain barrier becomes permeable. The organisms then leak out of the brain but are insufficient to cause an effective antigenic response in the time available (Berenbaum et al, 1960).

The majority of fresh isolates of B. pertussis have low IC virulence (Andersen and Bentzon, 1958; Iida et al, 1962). However, ^{LOW}virulence strains behave as high virulence strains in the first 24 h of infection (Adams, 1970). However with low infecting doses the brains became sterile within 5 days whereas high infecting doses of low virulence strains killed the mice by Day 3.

Adams (1970) found the same immediate 90% loss of organisms as Dolby and Standfast (1961) and suggested this was due to washback along the needletrack. Complement appeared to have no part in the defence of the brain since low virulence strains did not have enhancement of infection in mice deficient in C5 component. Modulation induced by growth of challenge cultures at 25°C was ruled out as a possible reason for loss in virulence. An interference phenomenon was observed when a mouse infected with a low virulence strain

was reinfected with a high virulence strain. The mice survived. Adams (1970) proposed this was due to the infection sites being already occupied.

4.1.4 Effect of vaccination

Berenbaum et al (1960) examined the histological changes in the brains of vaccinated and challenged mice. The cellular reaction of day 1 varied little from that in unprotected animals. Polymorphs were the predominant cell together with isolated lymphocytes and macrophages in the meninges. By day 6 mononuclear cells, mainly lymphocytes but also macrophages, accounted for the intense inflammatory reaction in the meninges and choroid plexus. Plasma cells were also present and actively multiplying. This reaction was still present after 2 to 3 weeks and on the 6th week cellular infiltration was still apparent.

Berenbaum et al (1960) also found that the multiplication of the B. pertussis was similar in vaccinated and unvaccinated mice until day 3 of infection. Thereafter the infection peak of the vaccinated animal occurred on day 4 with around 10^5 organisms. Subsequently the number of organisms declined until day 5 or 6 when the infection was reduced to 10^3 bacteria.

4.1.5 Effect of passive immunisation

Passively protected mice given antisera i.p. from 3 days before to 3 days after IC challenge all started to recover 3-4 days after infection. Both actively and passive immunization were effective only after the blood brain barrier was broken (Berenbaum et al, 1960).

Spasojevic (1962) and Holt (1972) administered antisera and infecting organisms together in a mixture. The same antisera to B. pertussis in tissue culture studies prevented adherence of the bacteria to human fibroblasts, although these sera would not detach previously adherent cells. This ability correlated with the passively protective activity of the antisera by the IC/mix route.

4.2 Intranasal infections

4.2.1 Modes of administration

(i) Aerosol spray

Sato et al (1980) claim to have uniformly infected mice by exposing them for 30 min to aerosols generated from bacterial suspensions of 10^8 and 10^9 organisms ml^{-1} . The mean bacterial count in the lungs of these animals infected with the respective 10^8 and 10^9 suspensions was 2.3×10^4 and 1.0×10^5 organisms. Twenty minutes after stopping, the mice were removed and placed in animal cages with air filter covers.

(ii) Intratracheally

By making a longitudinal incision over the ventral surface of the neck and removing fascia from the trachea, Bradford (1938) inoculated the organisms into the trachea via a small needle on a tuberculin syringe. The slit in the skin was healed by a small amount of collodion. Te-Punga and Preston (1958) showed that intratracheal inoculation gave more uniform administration of a lethal infection than IN instillation. Nembutal anaesthesia was used to prevent irregularity in breathing. However since volatile anaesthetics had been shown to increase the virulence of the infecting organism

(Te-Punga and Preston, 1958) the mice were treated with an ether/chloroform mixture 1 to 2 h before the i.p. injection of nembutal.

The intratracheal inoculum was given with a modified calibrated pasteur pipette which was slightly curved at the end and tapered with a small hole at the tip. The pipette was then passed over the base of the tongue and directed into the base of the trachea. Reproducible mortality rates were found over a series of lethal doses.

(iii) Intranasally

Burnet and Timmins (1937) anaesthetized mice with an ether/chloroform mixture until rapid, regular breathing was induced. The mice were then removed and placed in a fume cupboard. The organisms were instilled in a volume of 0.05 ml with the nasal axis in the vertical. A major drawback of the procedure was the bubbles which formed if the mouse was not breathing regularly.

4.2.2 Mouse strains and sex

Most of the early papers did not mention the mouse strain which was used and merely state "white mice of the laboratory" (Andersen, 1952; Fisher, 1955; Dolby et al, 1961). Named mouse strains in which a B. pertussis SLIN infection have been established are N:NIH(S.W.) (Geller and Pittman, 1973), Anglia (NIH) and HAML/CR (Pittman et al, 1980) and DDY and ICR (Sato et al, 1980; 1981).

Standfast (1951) examined the virulence of B. pertussis in 3 mouse strains and found a 3 to 5 fold difference in virulence. Pittman et al (1980) found HAML/CR mice overall more responsive than Anglia NIH as measured by recovery of B. pertussis by lung culture. Females of both strains were more susceptible to a lethal infection than males.

4.2.3 Mouse Age

Mouse age or weight is an important factor in the susceptibility to infection by the IN route in contrast to the IC route. Culotta et al (1938) showed a 93% mortality rate in 10-12 gram mice and a 66% mortality rate for 20-25 gram mice for the same infecting dose. Standfast (1951) confirmed these findings for two mouse strains. Pittman et al (1980) found age significantly influenced mortality, degree and duration of infection. The pathophysiological changes associated with infection were more marked and/or of longer duration in younger than in older mice. Sato et al (1980) found that older animals of the HAM 1/CR strain were less susceptible.

4.2.4 Strains of *B. pertussis*

A variety of *B. pertussis* strains have been used to establish IN infection (Table 4). Wide variations in virulence, as measured by the LD₅₀ of the organisms, were shown for freshly isolated strains even when the strains were subcultured only once after isolation before being used (Standfast, 1951). These freshly isolated strains showed a hundred fold difference in virulence. The rate with which the virulence of strains degenerated also varied. One strain showed a decrease in virulence of only 3/2 fold and another showed a 300 fold decrease over the same number of subcultures. The strains with high starting virulence showed a much lower rate of degeneration than the lower virulence strains. Enhancement of IN virulence by mouse passage was not successful.

Standfast (1951) also examined whether the virulence was influenced by the viability of infection. The LD₅₀ was calculated

in terms of total and viable count. Increased percentage viability of the infecting dose did not necessarily increase the virulence of the infection. Virulence of the fresh isolates was unaffected by toxin production and haemagglutinin content. There was no relationship between virulence and when organisms were isolated from the host, in relation to the onset of disease or the type of swab used to collect specimens (pernasal or postnasal).

4.3 Characteristics of intranasal infections

Intranasal infections are of two general types: lethal and sublethal. Individual authors' classification of these doses varies (Table 4). The dose used is dependent on susceptibility of mouse strain and the virulence of the B. pertussis culture.

4.3.1 Growth of organisms and host mortality

Fisher (1955) found the mean multiplication factor (the difference between the mean log viable count in the lungs and the log viable count of the infecting dose) decreased as the infecting dose increased (Table 5). The multiplication factor was negative with an infecting dose of 500×10^6 , ie there was a reduction in viable counts over the 7 days of infection. Dolby et al (1961) found the doubling time of viable counts increased as the infecting dose was increased, ie the multiplication rate was decreasing. The largest infecting dose again showed a decrease in viable count.

Dolby et al (1961) found actively immunized mice given a lethal infection of 10^7 organisms reduced the viable count to $< 10^4$ organisms in the lung within 4 days. Passively immunized mice

Table 4 : Comparative data from various sources relating to the infecting dose and mortality of mice given a respiratory tract infection with *B. pertussis*

Reference	Organism strain	Infecting dose and result			
		Lethal	% Mortality	Sublethal	% Mortality % Lung infection
North and Anderson (1942)	N.M.	50×10^6	100	N.M.	N.M. 85
Anderson and North (1943)	N.M.	50×10^6	100	5×10^3	10 90
North (1946)	N.M.	100×10^6	90-100	5×10^4 5×10^5	0 80 0 100
Proom (1947)	N.M.	100×10^6 10×10^6 10^6	100 90 40-70	8×10^4	0 80
Pittman (1951)	18-682	10×10^6 10^6	33-44 13-29	10^5 5×10^4	22 N.M. 10
Cooper (1952)	CNI34	250×10^6	100	5×10^6	N.M. N.M.
Fisher (1955)	FP12 and P81	500×10^6 50×10^6	49 28	5×10^6 5×10^5 5×10^4 5×10^4	N.M. 100 N.M. 50-81 40-60
Tepunga and Preston (1958)	GL353	250×10^6 25×10^6	60 50	N.D. ^a N.D. ^b	N.D.

Table 4 (contd.)

Reference	Organism strain	Infecting dose and result			
		Lethal	% Mortality	Sublethal	% Mortality
Dolby et al (1961)	C2621	$> 10^7$	100	$< 10^7$	0
	G353	$> 10^7$	100	$< 10^7$	0
Geller and Pittman (1973)	18-323	N.D.	N.D.	2.5×10^5	100
	18-323	2.2×10^6	93	2.2×10^5	6
Pittman et al (1980)		2.2×10^6	43	3.0×10^5	13
		1.35×10^7	36	1.35×10^5	2
		1.35×10^6	10		
Sato et al (1980)	Tohama	1.2×10^6	90	1.0×10^5	30
	Phase I			2.3×10^4	0

a N.M. = Not mentioned

b N.D. = Not done

Table 5 : Multiplication of *B. pertussis* in mouse lungs

Reference	Organism strain	Infecting dose	Peak of infection (Days)	Mean Generation Time
Proom (1947)	N.M. ^a	10×10^6	14	2 days
		5×10^4	14	1 day
Cooper (1952)	CN134	5×10^6	7-8	N.M.
Andersen (1952)	2 atoxic	10^5	10-24	N.M.
	5 atoxic	10^5	10-24	
	108	10^5	10-17	
Dolby <u>et al</u> (1961)	C2621	10^5	14-21	N.M.
		10^2	14	7.7 hr
		10^7	14	19.0 hr
Geller and Pittman (1973)	18-323	2×10^5	14	N.M.
Sato <u>et al</u> (1980)	Tohama Phase I	6×10^4	14	N.M.

a N.M. = Not mentioned

reduced a lethal infection of 10^7 organisms to a sublethal level by preventing multiplication to the critical number at which death would have resulted. Standfast and Dolby (1958) challenged passively immunized mice with a sublethal infection and the viable count was reduced to zero within 2 days.

The infection peak in sublethally infected mice varied from Day 7 (Cooper, 1952) to Day 24 (Andersen, 1952). Dolby et al (1961) found the G353 and C2621 had the respective infection peaks of D14 and D21. The time of infection peak depended on mouse and strain of organism (Table 5).

Pittman (1951) examined the mortality rate over a range of infecting doses (Table 4). She showed that a dose of 10×10^6 organisms gave 35-40% mortality and 50×10^3 organisms gave 10% mortality. The mean time of death was 16.1 days and 23.5 days for the respective doses. Dolby et al (1961) characterised the in vivo growth curves over a range of infecting doses for the two strains C2621 and G353. An infecting dose of $< 10^7$ organisms resulted in no mortality. Above this critical number of organisms a lethal infection occurred with 100% mortality. These lethal infections occurred when growth was sufficient to reach 10^8 organisms before 10-14 days. Standfast and Dolby (1961) calculated the mean time of death for the range $1/100 \text{ LD}_{50}$ to 10^5 LD_{50} of G353 strain moved from 15 days to 1-2 days.

4.3.2 Pathophysiological changes after infection

(i) Histological

The histological changes occurring in a lethally infected mouse have been well documented. Red patches appear all over the

lung by Day 3 of infection (Bradford, 1938). This colour gradually changes to a greyish-pink consolidation (Bradford, 1938; Burnet and Timmins, 1937). Acute oedema was found in the trachea and exuding from the consolidated areas of a dying mouse. Portions of the lungs are airless (Burnet and Timmins, 1937).

Cellular infiltration of mononuclear and polymorph cells developed by Day 3 of infections with macrophage infiltration slowly replacing the polymorphs, (Bradford, 1938). Organisms were present mainly in the bronchioles but also in the bronchi and rarely on the trachea. Massive proliferation of the bacilli could be seen in the mucus with slight destruction of the cilia and ciliated surface to form necrotic areas in the epithelium which also permitted growth (Burnet and Timmins, 1937). Bradford (1938) recovered B. pertussis from spleen, heart and bloodstream. This is an unusual result as subsequent experience suggested pertussis is a localized infection of the lungs (Proom, 1947).

Cheers and Gray (1969) examined the development of histopathological changes in mice after a sublethal pulmonary infection with B. pertussis. Lung sections showed bacteria in the mucus of bronchioles early in infection and in the alveoli by Day 7. Polymorphs and macrophages were present in equal proportions on Day 11. On Day 14 a polymorph invasion led to a massive reduction in bacterial numbers in the bronchioles. The polymorphs then disappeared but macrophages containing bacterial cells persisted to about the 6th or 7th week.

Muse et al (1977) and Sato et al (1979) presented electron micrographs showing B. pertussis on the ciliated epithelium of a

guinea pig and mouse lung respectively. Sato observed that some organisms are found on non-ciliated epithelium although most were associated with ciliated cells.

(ii) Opsonocytophagic

Cooper (1952) examined the opsonocytophagic indices of normal and infected mice 8 days after a sublethal infection with B. pertussis. The opsonocytophagic index is the ratio of organisms within macrophages from a normal mouse and macrophages infected mice. The index for normal to infected mice was 1:20.

Cheers and Gray (1969) observed the change from extracellular multiplication of the bacteria after a few days to intracellular existence within alveolar macrophages. The latter had increased killing power but normal phagocytic capacity. However many bacteria survived within the macrophages and this steady state where the number of viable organisms did not change was termed the complaisant phase.

(iii) Leucocytosis

Bradford (1938) examined leucocytosis in lethally infected mice over a period of 15 days. There were two control groups consisting of uninfected mice and mice infected with atypical B. pertussis. By day 5 B. pertussis infected animals showed leucocyte counts between $39,000 \text{ mm}^{-3}$ and $220,000 \text{ mm}^{-3}$. Uninfected mice had counts of $20,000 \text{ mm}^{-3}$ and atypically infected animals gave counts of $6,500 \text{ mm}^{-3}$ to $62,000 \text{ mm}^{-3}$. By day 10 the leucocytosis persisted only in the typical B. pertussis infected mice. Cooper (1952) examined the red cell and leucocyte count of sublethally infected animals. The red cell count remained unaffected but the leucocyte count showed a five fold increase after 8 days.

Sato et al (1980) observed a peak WBC count of 1.95×10^5 at Day 14 of infection in sublethally infected mice, after which the count remained steady. Pittman et al (1980) reported peak leucocytosis at Day 10 of a lethal infection and Day 20 of a sublethal infection. Leucocytosis and hypothermia were highly correlated in lethally infected mice as also was the body and spleen weight.

(iv) Histamine sensitisation

Pittman (1951) demonstrated histamine sensitivity after a sublethal infection and also determined the onset and duration of this sensitisation after 2 infecting doses of 10×10^6 and 10^6 organisms. The larger infecting dose gave peak sensitivity on day 10 of infection persisting to day 50 with 100% mice showing sensitivity. The lower dose gave a slower development of peak sensitivity probably due to longer multiplication time required for the infecting dose to reach infection peak. Peak sensitisation occurred on day 15 with 70% of the mice showing sensitivity. The degree of sensitivity for the lower dose was not as great as the larger dose; however both infections gave a sensitivity which lasted beyond 50 days.

Lung culture and lung pathology examination showed a correlation between development of sensitivity and development of infection. Characteristic lung pathology was found in 83% of sensitised mice and 64% had positive lung culture. The duration of sensitivity however was much greater than persistence of lung infection. The organisms were rarely isolated beyond day 30, whereas sensitivity was demonstrated on day 50 for 33-50% of mice.

Geller and Pittman (1973) found the development of histamine sensitivity to peak at day 15-20 and to last well beyond day 50 after

a sublethal infection. Sensitisation was greater after an IN infection with live organisms than an IN instillation of dead organisms or an intraperitoneal injection of live or dead organisms. The i.p. injection of 2.5×10^5 organisms showed a slow developing sensitivity with a peak at day 20 compared to a peak at day 4 to 5 for an i.p. injection of 10^9 organisms. A possible explanation would be a different mechanism of sensitisation occurring with the different doses.

Pittman et al (1980) reported peak sensitivity of day 20 and 30 for mouse strains HAML/CR and Anglia NIH respectively. Although data were insufficient to make conclusive correlations, it seemed that histamine sensitivity was correlated to infectivity, IRI^a concentrations and the ratio of spleen to body weight.

^aIRI = Immuno reactive insulin.

4.3.3 Immunity to infection

(i) Acquired by infection

Cooper (1952) examined the immunity which developed after a SLIN infection by 2 methods: (i) Reinfection with LIN infection after 4 weeks and (ii) IC challenge after 4 weeks with doses ranging from 5 LD₅₀ to 100 LD₅₀. Mice reinfected by the IN route had a 93% survival rate. Lung cultures were not made. Resistance to IC challenge was greatest with low LD₅₀ and immunity was maximum 4 weeks after infection with 80% survival rate against 5-9 LD₅₀ and 58% survival rate against 50-100 LD₅₀ challenge. Immunity to IC challenge was not demonstrable after 6 weeks.

Andersen (1953b) reinfected mice with a SLIN challenge after 10 wk. Lung cultures were usually negative by day 4 in reinfected mice. Control mice still had positive lung cultures at day 26 of infection. However it was not stated whether the control mice were the same age as the reinfected animals. If the control mice were younger the comparison would be invalid, and the inability to establish infection in the convalescent mice might have been due to the older animals having an age-related resistance rather than specific immunity. Development of serum agglutinins occurred around day 38. Andersen concluded that acquired resistance after infection was due to systemic as well as local immunity.

(ii) After vaccination

Burnet and Timmins (1937) showed that mice immunized with live cultures of B. pertussis had a significantly higher degree of protection against IN challenges of 10×10^6 or 100×10^6 organisms than mice immunized with formalin-treated cells. Neither vaccine was effective against a challenge of 1000×10^6 organisms. No difference in vaccine potency was found when administered either by the i.p. or s.c. route.

Proom (1947) demonstrated that an i.p. challenge of live B. pertussis after vaccination was ineffective for assay of vaccine potency. B. pertussis does not multiply in the blood or peritoneal cavity of mice and death after an i.p. injection of living organisms is due to toxæmia. This does not seem to be relevant to pulmonary infections, where antibacterial immunity is believed to be the major immune response in clearing infections.

Andersen (1953b) compared results of vaccine potency obtained by IC and SLIN challenge. Three vaccine doses were examined:

120×10^6 , 600×10^6 and 3000×10^6 organisms. The largest total dose gave the greatest survival rate after IC challenge and the quickest clearance of infection from lung after SLIN infection. Correlation was found between vaccine potency measured by the IC route and SLIN route of infection. Administration of vaccine by the i.p. gave better protection than the s.c. route.

Fisher (1955) compared the efficacy of protection of 3 vaccines, boiled and unboiled, as measured by 3 different challenges: IC, LIN, SLIN infections. A strong correlation was found when vaccine potency was measured by the IC and SLIN routes of infection. The protective antigen against both IC and SLIN challenge was found to be heat labile (100°C). Survival after LIN challenge showed no correlation with the other routes of infection and also the protective antigen by this route was heat stable (100°C).

Andersen and Bentzon (1958) also compared vaccine potencies as measured by the IC and SLIN challenge procedures. Using the same highly mouse virulent strain for both routes of infection similar results were obtained. However SLIN challenge results had greater variation in protective potency of vaccine when freshly isolated strains were used. The mice were infected at 5 weeks and therefore were less susceptible to infection than at the normal age for giving SLIN infection. Standfast and Dolby (1961) also confirmed a correlation between vaccine potency measured by IC and SLIN route of infection which did not correlate with the LIN infection.

Intranasal vaccination has been investigated. North and Anderson (1942) found it to be more effective than i.p. vaccination when mice were challenged with 50×10^6 infecting dose 7 days after

vaccination. Intranasal vaccination showed an elevated non specific local immunity against organisms with antigenic relationships to B. pertussis, ie H. influenza and B. parapertussis. Histological examination of lungs of immunised mice infected with these organisms showed a marked cellular infiltration. However this comparison between vaccination routes might not have been strictly valid since 14 days, not 7, is the usual time interval allowed for systemic immunity to develop. Complete development of general immunity would not have occurred in the i.p. vaccinated mice.

Cooper (1952) was unable to demonstrate immunity after IN vaccination when he infected mice by the IC route over a 5 week period. However Andersen (1953) demonstrated general immunity after a total IN vaccine dose of 2000×10^6 organisms in that the mice were able to survive an IC challenge. However IN vaccination was more dependant on vaccinating dose than i.p. or s.c. vaccination. Dolby et al (1961) found vaccination by the IN route had a PD_{50} of 1650×10^6 compared to PD_{50} of 190×10^6 for the i.p. route.

(iii) After passive immunization

North et al (1939) examined the protective value of a range of i.p. injected sera against IN infections. Sera from adult contacts gave best protection against a LIN infection. Convalescent and immunized children's sera were equally effective in lung clearance. Evans (1940) found he could remove antibacterial activity from rabbit anti-B. pertussis sera and leave anti-HLT. However Anderson and North (1943) found no anti-HLT activity in adult contact sera which led them to believe that anti-HLT was of little value in IN infections. Death after an i.p. injection of H. pertussis was

prevented by anti-HLT but not by antibacterial serum (Proom, 1947). However an i.p. injection of antibacterial serum prevented a SLIN infection. Antitoxin had no effect when given by the i.p. route.

Evans (1944) demonstrated protective value of intranasally introduced antitoxin when mixed with the infecting dose. There was a 40% reduction in infected lungs and 100% reduction in mortality. Thus anti-HLT appeared to be protective when at the site of infection. North (1946) concluded that antibacterial sera was almost as effective as anti-HLT sera when given intranasally 24 h before IN infection.

When antisera were given 24 h before IN infection, antibacterial sera was of greater protective value than anti-HLT sera (Proom, 1947). Phase I and IV sera antibacterial sera and horse hyperimmune sera were all shown to protect against IN challenge of 50×10^6 organisms. Immune sera given 14 days after infection dramatically reduced growth. Within seven days the lung culture of most mice was negative. However Proom could only demonstrate passive protection of antisera against SLIN and not against LIN infection.

Sato et al (1981) found that the i.p. injection of anti-LPF and anti-F-HA before aerosol challenge protected mice, as evidenced by survival and normal rate of body weight gain.

4.3.4 Nature of immune response

North (1946) demonstrated protective antibodies in mouse sera 30-50 days after SLIN infection. Andersen (1953b) found that the onset of serum agglutinins and protective antibodies to reinfection were simultaneous around day 40. However serum

agglutinins were not always present in sera that were passively protective. Cooper (1952) also found protective antibodies against intranasal reinfection by day 28. However both these results may be influenced by the age of the mouse at reinfection rather than by a state of specific immunity. No passive protective antibodies or agglutinins were present on Day 15 (North, 1946; Andersen, 1953b). Complement-fixing antibodies were not present on day 8 of infection (Cooper, 1952).

Geller and Pittman (1973) examined the classes of immunoglobulins present in the tracheobronchial washings (TBW) and sera of SLIN infected mice. An increase in B. pertussis specific IgA, IgG, IgG_{2a} and IgG_{2b} was shown in the TBW, beginning on day 15 and persisting to day 50. The serum immunoglobulin did not show such a marked change except for IgA which increased on day 30. The appearance and gradual increase of Ig in the lung corresponded to the disappearance of organisms between 20 to 30 days. Pittman (1976) investigated the importance of IgA in the clearance of organisms from the lung. She found IgA concentrations in convalescent mouse sera increased in parallel with the passive protective activity of similar sera to LIN murine infections (the latter had been previously demonstrated by Winter, 1953).

OBJECT OF RESEARCH

Pertussis, or whooping cough is still a very poorly understood disease, despite the elapse of over 70 years since Bordet and Gengou isolated the causative organism Bordetella pertussis.

In common with many human pathogens, B. pertussis while transmissible to laboratory animals, does not set up an infection which fully reproduces all the features of the human disease.

Recently there has been reawakened interest in the B. pertussis intranasally-infected mouse as providing a useful experimental model of pertussis in man. By choice of appropriate infecting doses, mice can be given either a lethal or a sublethal pulmonary infection by the IN route. It is the sublethal pulmonary infection of the mouse which provides the subject for this investigation.

The object of the investigation was to determine the nature of the immune response which develops during the course of the murine sublethal pulmonary infection and to relate it as far as possible to what is known about the human disease.

MATERIALS AND METHODS

SECTION I : STRAINS, MEDIA AND CULTURAL PROCEDURES

1.1 Strains

Three Phase I strains and one Phase IV strain of B. pertussis were used.

Strain 18-323 Phase I was obtained from two different sources:

- (i) Dr. M. Pittman of the Bureau of Biologics, Food and Drug Administration, Bethesda, Maryland, U.S.A. This strain is referred to as 18-323 (P).
- (ii) Dr. F. Sheffield of the National Institute for Biological Standardization and Control, Hampstead, London. This strain is referred to as 18-323 (S).

Strain 18-334 was a Phase I vaccine strain from the Connaught Laboratories Limited, Toronto.

Strain 77/18319 was a Phase I fresh isolate supplied by Dr. R. Fallon, Ruchill Hospital, Glasgow.

The Phase IV strain was:

Strain D30042 from Dr. J.M. Dolby of the Lister Institute of Preventive Medicine, Elstree, Herts.

All strains were maintained freeze-dried and also frozen after suspension in liquid nitrogen in a fluid containing 1% Casamino Acids (CAA) and 10% glycerol.

1.2 Media

The following were used for the cultivation of B. pertussis:

Bordet-Gengou agar (BG). The agar base was obtained from

Gibco Bio-cult Diagnostics Ltd., Paisley, Scotland, and after rehydrating and autoclaving was mixed at 45°C with defibrinated horse blood from the same source to give 17% (v/v) blood.

A modified Cohen and Wheeler medium (Sato and Arai, 1972).

A modified Hornibrook medium (Parton and Wardlaw, 1975).

The composition and preparation of these media are given in Appendix 1.

1.3 Growth of cultures

Freeze-dried cultures were reconstituted with 1% (w/v) CAA (Appendix 1) and grown on B.G. for 72 h at 35°C in a plastic box containing a beaker of water to saturate the atmosphere. Liquid nitrogen suspensions were left to thaw at room temperature and grown on B.G. for 24 h at 35°C.

1.3.1 Challenge cultures

Liquid nitrogen suspensions were inoculated onto B.G. and incubated at 35°C for 24 h. These were then subcultured on B.G. and incubated at 35°C for 24 h.

1.3.2 Batch cultures

One millilitre of 100 opacity units (o.u.) suspension of B. pertussis 77/18319 was inoculated into Roux bottles, laid flat, containing 100 ml modified Cohen and Wheeler medium and incubated at 37°C for 5 days without shaking. The suspension was then centrifuged at 10,000 rpm for 30 min at 4°C to obtain culture supernate.

1.4 Standardisation of bacterial concentration by opacity

The concentration of bacterial suspensions was estimated by comparison with the International Opacity Reference Preparation supplied by the World Health Organisation International Laboratory for Biological Standards (National Institute for Biological Standards and Controls, Holly Hill, Hampstead, London). This standard is defined as having 10 o.u. and consists of a plastic rod mounted in a 6 x 5/8 test tube. This and the bacterial suspension were matched by viewing a printed card through both tubes until the density of the bacterial suspension equalled that of the opacity rod (Perkins et al, 1973). The dilution factor used to obtain matching of the cell suspension with the reference preparation was calculated and multiplied by 10 to give the concentration of the undiluted bacterial suspension in opacity units (o.u.).

A concentration of 10 o.u. was found to be equivalent to approximately 2.5×10^9 ^{ML⁻¹} colony forming units (c.f.u.) of a 24 h culture of B. pertussis strain 18-323 freshly harvested from BG.

1.5 Challenge suspensions of B. pertussis strain 18-323

Twenty-four hour growth of B. pertussis strain 18-323 was scraped off B.G. into C.A.A. and matched against the International Opacity Reference Preparation. The matched 10 o.u. suspension (2.5×10^9 c.f.u. ml⁻¹) was diluted by a factor of 1000 in dilution steps of 10 to give the challenge suspension for administration to mice. The bacterial concentration was therefore 2.5×10^6 c.f.u. ml⁻¹ and each mouse received 0.05 ml or 1.25×10^5 c.f.u.

The challenge suspension for an intracerebral infection was 10^6 c.f.u. ml⁻¹ and each mouse received 0.03 ml, or 3×10^4 c.f.u.

The viable count of each challenge suspension was determined by making 10 fold dilutions in CAA and spreading 0.05 ml on each of 2 BG plates which were incubated at 35°C for 5 days, when the colonies were counted.

SECTION II ANIMALS AND ANIMAL PROCEDURES

2.1 Mice

Mice were from a randomly-bred closed colony originally derived from the HAM 1/CR strain (Charles River, U.K. Ltd., Manston Road, Margate, Kent). They were kept in a room at 22°C in a 12 h light cycle and maintained with standard pelleted diet on peat litter and with water ad lib.

2.2 Administration of fluids intranasally

The method of Burnet and Timmins (1937) was used for the IN infections except that ether alone, rather than an ether/chloroform mixture, was used as the anaesthetic. Mice, 3-4 wk old and of either sex were placed in groups of up to 5, in a glass jar fitted with an ether-soaked pad in the lid. They were kept in the jar until they were unconscious and were breathing deeply and at a regular rate. They were then removed from the jar, held so that the nose was vertical and the challenge fluid administered by placing 2 drops of 0.025 ml volume from a calibrated dropping pipette (Dynatech, Kloten,

Switzerland). The drops were usually inhaled rapidly but if the breathing was irregular spluttering occurred. After recovering from anaesthesia, which took around 2 min, the mice were then colour coded by rubbing blue, red or yellow dye on head, back or rump and put back into their cages. The whole process of challenging a batch of mice was completed within an hour so that the bacterial suspension did not age unduly from beginning to end.

2.3 Administration of challenge suspension intracerebrally

Mice, 3-4 wk old and of either sex, were anaesthetised (as in 2.2) and were injected IC with 0.03 ml of challenge suspension containing approximately 3×10^4 cfu B. pertussis 18-323 (P) or (S) organisms. The challenge was given at right angles to the skull, through the parietal bone and using a 26 x 5/8 needle and 1 ml syringe.

The virulence of the challenge suspension was titrated by injecting groups of mice with dilutions 1/10, 1/50 and 1/250 by the same technique.

Again the mice were colour coded with similar dyes after recovery from the anaesthetic. Intracerebral injections were always completed within 90 min of harvesting the cells.

2.4 Collection of serum

The mice were anaesthetised individually in an ether jar, then secured to an operating board and kept under ether anaesthesia with a small beaker containing a cotton wool pad soaked in ether

held over the head. A 2 ml syringe with a 25 gauge 5/8" needle was used to withdraw blood by heart-puncture through the rib cage. The volume of blood usually obtained was 1.5 ml. Normally, the bloods from a large number of mice were pooled and allowed to clot in a universal container, for up to 5 hr at room temperature before separating the serum by two successive centrifugations at 3,000 rpm for 15 min. The cell free supernatant serum was removed and dispensed into 2 ml aliquots and kept at -70°C .

2.5 Collection of tracheobronchial washings (TBW)

After each heart-punctured mouse had died as a result of exsanguination, a longitudinal incision was made in the ventral surface of the abdomen and the skin and fatty tissue cleared from around the trachea. The rib cage was opened to allow the lungs to expand freely. The interior of the lungs was then washed out by making a small incision in the wind-pipe and flushing in 1 ml of sterile saline via a cannula made from constricted plastic tubing and attached to a 2 ml syringe. After 30 sec, the fluid (usually about 0.75 ml) was withdrawn, and pooled in a universal container held on ice. The pooled TBW were centrifuged at 10,000 rpm for 30 min, dispensed into 2 ml aliquots and kept at -70°C . All TBW were millipore filtered ($0.45\ \mu\text{m}$) before use in case viable B. pertussis was present.

2.6 Removal of blood for leucocyte count

Mice were anaesthetised (as in 2.2) and bled from the orbital plexus with heparinized capillary tubes (Harshaw Chemicals Ltd.,

Daventry, Northhants, England). The blood sample (40 μ l) was immediately transferred with a Coulter pipette to plastic vials containing 20 ml Isoton II diluent (Coulter Electronics Ltd., Harpenden, Herts, England) to give a final blood concentration of 1:501 (v/v).

Immediately before counting, six drops of Zap-Oglobin (Coulter Electronics Ltd., Harpenden, Herts, England) were added to each vial to lyse the red blood cells. The leucocyte were counted in a Coulter Counter model FN (Coulter Electronics Ltd., Harpenden, Herts, England). Duplicate counts of each diluted blood sample were made and the mean of the counts ml^{-1} was calculated for each sample. Mean background counts were subtracted from each mean. To correct for coincidence losses during counting, mean counts above 10,000 were adjusted with a coincidence correction chart.

2.7 Histamine sensitisation

Normal mice, convalescent mice and mice given graded doses of pertussis vaccine were tested for histamine sensitivity by being injected intraperitoneally with 3 mg of histamine dihydrochloride (Sigma Chemical Co., St. Louis, Mo., U.S.A.) contained in 0.5 ml of saline. Survivors were counted 2½ h afterwards.

2.8 Autopsy

Mice were killed by dislocation of the neck, dipped in a beaker of disinfectant (hibitane) and pinned out on a board. The thorax was opened with sterile scissors and forceps (sterilized by

dipping in alcohol and flaming) and the lungs inspected for signs of consolidation, ie greyish-coloured areas. Healthy lungs were scored as 0, doubtful were scored as 1. Definitely infected lungs were scored from 2, where there were a few areas of consolidation, examples of which are shown on Plate 1, to 4 for complete or almost complete consolidation in all lobes of lungs. These gradations are illustrated in Figure 2.

A small portion of lung from the smallest of the right hand lobes was also taken with sterile scissors and forceps and the cut surface smeared onto a BG plate. After 4-5 d of incubation at 35°C, "culture scores" were allocated for the resultant B. pertussis growth as follows: 0 for no growth; 1 for 1-9 colonies; 2 for 10-99; 3 semi-confluent and 4 confluent (the criteria established by North, 1946). In both lung pathology and culture scores values greater than or equal to 2 were considered to represent a significant departure from the normal.

2.9 Subcutaneous inoculation of suckling mice

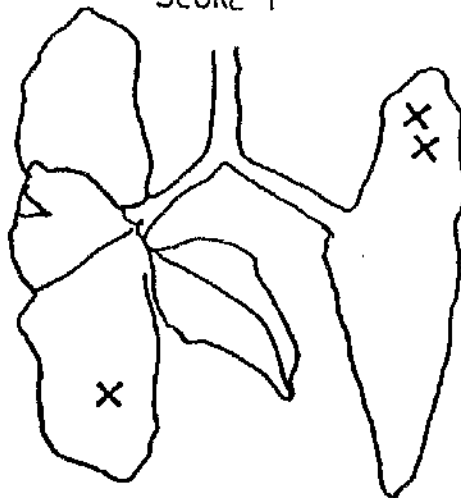
As an assay of HLT, fluid was injected subcutaneously into the back of the neck of 4 day old suckling mice of either sex in 0.05 ml volumes using a 1 ml syringe and 25 x 5/8 needle. The mouse was held between the thumb and forefinger face down on the bench. Care was taken to wear surgical gloves when handling suckling mice to minimise any smell of the handler being left which might lead to maternal rejection. After injection, the mouse was colour-coded with blue dye on the back, rump or left or right leg and returned to the litter as soon as the dye had dried.

Plate 1. Examples of mouse lungs with pathology score 2.

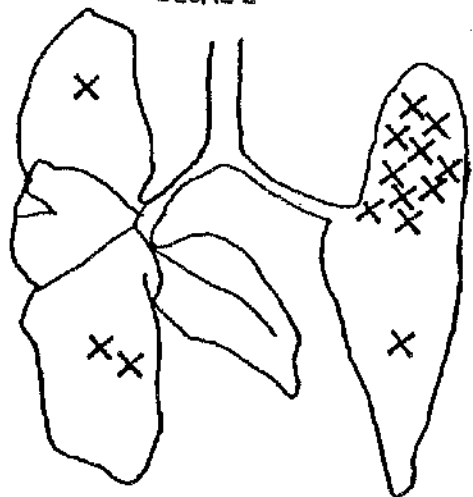


Figure 2. System of pathology scores in lungs of B. pertussis
infected mice. Areas of consolidation are shaded.

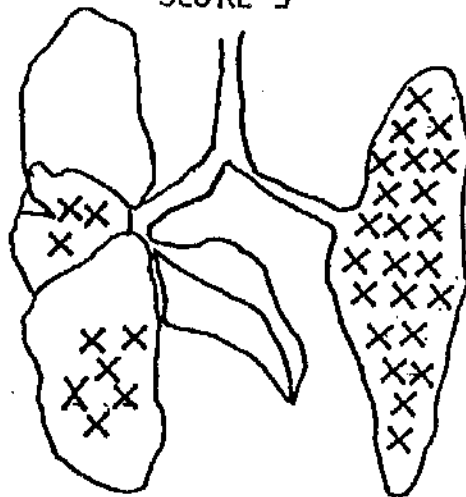
SCORE 1



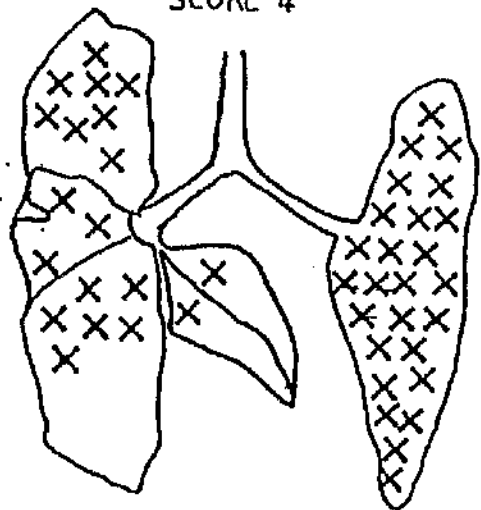
SCORE 2



SCORE 3



SCORE 4



SECTION III

BIOCHEMICAL PROCEDURES3.1 Protein estimation

The composition of the reagents is given in Appendix 2. The protein concentration of TBW was measured by the method of Herbert, Phipps and Strange (1971) with bovine serum albumin (BSA) as the standard.

- (i) A volume of 0.5 ml of distilled water was pipetted into duplicate tubes as reagent blanks.
- (ii) Samples were tested at undiluted and 1/5 dilution in 0.5 ml volumes in duplicate tubes.
- (iii) The BSA standard protein was diluted to give concentrations of 500, 400, 300, 200, 100 and 50 $\mu\text{g ml}^{-1}$. Volumes of 0.5 ml of each dilution was pipetted into duplicate tubes.
- (iv) All tubes received 0.5 ml 1.0N NaOH, boiled for 5 min, removed and allowed to cool.
- (v) All tubes received 2.5 ml of Reagent C and vortex mixed.
- (vi) After 10 min incubation at room temperature, 0.5 ml of Reagent D was added to each tube and mixed immediately.
- (vii) The tubes were then left at room temperature for 30 min for full colour to develop.
- (viii) The optical density was measured at 750 nm, each sample being measured against the reagent blank.
- (ix) A standard curve of OD_{750} versus BSA concentrations was plotted and the protein concentrations of TBW determined by interpolation on to that curve.

3.2 Lysozyme assay

The method was the Lysoplate procedure modified from that described by Osserman and Lawlor (1966). Lysoplate preparation is given in Appendix 3.

Doubling dilution of hen egg-white (H.E.W.) lysozyme (Sigma, London) over the range 40 to $0.3125 \mu\text{g ml}^{-1}$ were prepared in 0.15M phosphate buffered saline pH 6.4 as were doubling dilutions of pooled day 15 TBW. Aliquots of each dilution together with buffer controls were applied in duplicate to each lysoplate system, incubated at 20°C for 20 h and the diameters of the resultant zones of clearance recorded. This showed that pooled day 15 TBW gave a response parallel to H.E.W. lysozyme over the same dilution range.

Subsequently assays of TBW were made with H.E.W. lysozyme as standard.

3.3 Method for concentrating TBW

Pooled TBW from convalescent mice, eg a volume of 40 ml, was millipore filtered (Pore size $0.45 \mu\text{m}$, Millipore, Millipore S.A., 67120 Molsheim, France) to remove particulate matter and then placed in dialysis tubing (Medicell International, Liverpool Road, London) which had been boiled twice in saline and well rinsed. The tubing was then put in a plastic box which had Calbiochem Aquacide III (MW c.a. 20,000, C.P. Laboratories Ltd., Bishops Startford, Herts) as a layer on the bottom. The tubing was covered with aquacide III and allowed to stand for 3 h, when fresh aquacide was layered over. After a further 2 h, the aquacide was removed from the outside of the dialysis

tubing and the tubing washed thoroughly. Typically a 10-fold concentration was achieved.

SECTION IV IMMUNOLOGICAL STANDARDS AND PROCEDURES

4.1 Standard Vaccine

The standard vaccine was a freeze dried preparation of B. pertussis 18-334 in an ampoule containing 220 o.u. ml, ie 2.2 ml of 100 o.u., and was prepared in this department by A.C. Wardlaw and R. Parton in November 1977. It is referred to as Glasgow Standard Pertussis Vaccine.

4.2 Standard Antisera

(i) The U.S. Standard Antipertussis Serum (rabbit concentrated) Lot No. 2 supplied by C.R. Manclark (Division of Biologics Standards National Institutes of Health, Bethesda, Maryland, U.S.A. was the only standard antiserum used in all the immunological assays.

(ii) Rabbit antiserum prepared in this department by Dr. I. Livey against toxoided B. pertussis strain 134 supernatant lysate and containing 120 antitoxin units ml⁻¹ was also included as a known positive standard in the neutralisation of HLT haemorrhagic activity in suckling mice. Particulars of this material are given in the Ph.D. thesis of I. Livey (1982).

(iii) Rabbit antiserum prepared by J. Hertz (Hvidovre Hospital, Copenhagen, Denmark) raised against X mode 18-334 B. pertussis was incorporated in the back gel when determining the precipitin response by quantitative immunoelectrophoresis.

4.3 Inactivation of IgM and IgE

IgM in pooled convalescent sera and 10x concentrated TBW was inactivated by adding 0.2 ml to 1.6 ml saline and 0.2 ml 1.0M mercapto-ethanol. The mixture was incubated at room temperature for 1 h. The sample mixture was then made to the required dilution and used immediately (Nowotney, 1969).

IgE from pooled, convalescent serum at 1/5 dilution in saline and 10x concentrated TBW at 1/5 dilution in normal serum was inactivated by heating at 56°C for 4 h. The sample was then stored at 4°C until needed. This was the method used by Wardlaw et al (1979) to remove IgE from sera.

4.4 Removal of IgG and IgA from pooled serum and TBW

The IgG and IgA were removed from convalescent serum and TBW by precipitation with the corresponding goat anti-mouse serum, ie anti-mouse IgG (heavy and light chains) and anti-mouse IgA (alpha chain specific) of Cappel Laboratories, Inc., Cochranville, P.A., 19330, U.S.A.

4.4.1 Titration of optimum concentrations for immunoglobulin in class precipitation from pooled serum and TBW

The titration to find the optimum concentrations of goat anti-mouse serum and convalescent samples which would precipitate the Ig classes was done by immunodiffusion. The composition of reagents is given in Appendix 4. Clean glass slides (10 x 50 mm) were placed on a levelling tray and 5 ml of molten 0.75% barbitone agar were

spread evenly over the slide with a pipette. Wells of 3 mm were cut in paired rows with 1 cm between the adjacent wells and 0.5 cm from the opposite well.

The range of dilutions in the titrations of reactants and their optimal concentrations were as follows.

- (i) Goat anti-mouse IgG at 1/5, 1/10 and 1/20 dilutions and pooled day 61 convalescent sera at 1/50, 1/100, 1/200 and 1/400 dilutions (PLATE 2).
- (ii) Goat anti-mouse IgG at 1/5, 1/10 and 1/20 were used and the 10x concentrated pooled day 48 convalescent TBW at 1/5, 1/10, 1/20 and 1/40 dilutions (PLATE 3).
- (iii) Similarly goat anti-mouse IgA at dilution 1/5, 1/10 and 1/20 and pooled day 61 convalescent serum at 1/5, 1/10, 1/20 and 1/40 dilutions (PLATE 4).
- (iv) Finally goat anti-mouse IgA at dilutions 1/2, 1/4 and 1/8 and 10x concentrated pooled day 48 at neat 1/2, 1/5 and 1/10 dilutions (PLATE 5).

The four plates were held for 18 h at room temperature, washed, stained and lines of precipitation recorded. An arrow on each plate indicates the precipitation line where it appeared the reactants were roughly at optimal dilutions.

4.4.2 Precipitation of IgG and IgA from pooled sera and TBW

Having determined the dilution range within which optimal precipitation proportions of goat anti-mouse sera and pooled convalescent mouse sera and TBW occurred, the next step was to perform the precipitations over a dilution range to determine optimal dilutions of the various reactants. This was done by mixing equal volumes of reactants (dilutions are given in Table 6) and incubating the

Plate 2 Determination of the range of concentrations of pooled
day 61 convalescent sera and of goat anti mouse IgG
used to precipitate IgG

Anti IgG ^a Dilutions	$\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$
	0	0	0	0	0	0
	0	0	0	0	0	0
C.S. ^b dilutions	1/50			1/100		

Anti IgG dilutions	$\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$
	0	0	0	0	0	0
	0	0	0	0	0	0
C.S. dilutions	1/200			1/400		

a Anti IgG = Goat anti mouse IgG

b C.S. = Pooled day 61 convalescent sera

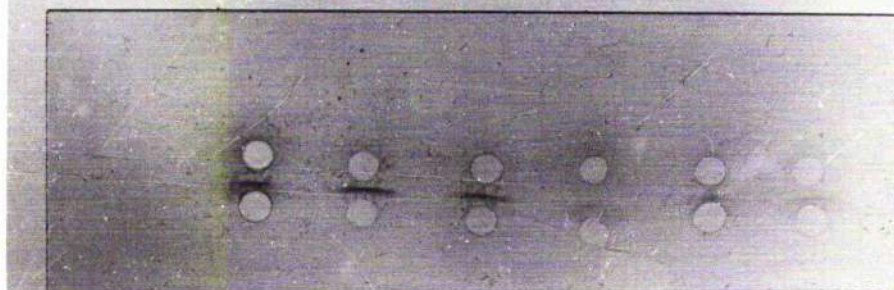
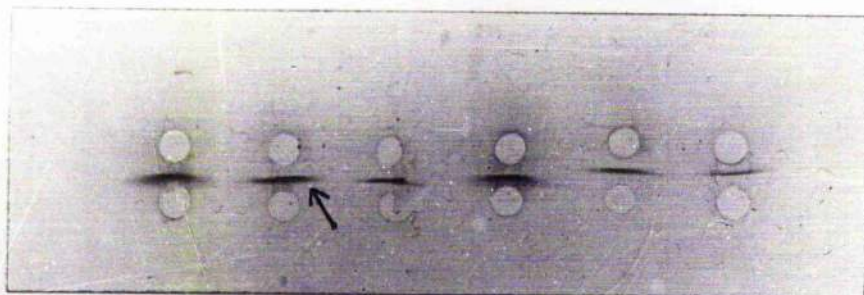


Plate 3 : Determination of the range of concentrations of 10x
concentrated pooled day 48 convalescent TBW and of
goat anti mouse IgG used to precipitate IgG

Anti IgG ^a	$\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$
	0	0	0	0	0	0
	0	0	0	0	0	0
C.TBW ^b	1/5			1/10		

Anti IgG	$\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$
	0	0	0	0	0	0
	0	0	0	0	0	0
C.TBW	1/20			1/40		

a Anti IgG = Goat antimouse IgG

b C.TBW = concentrated pooled day 48 convalescent TBW

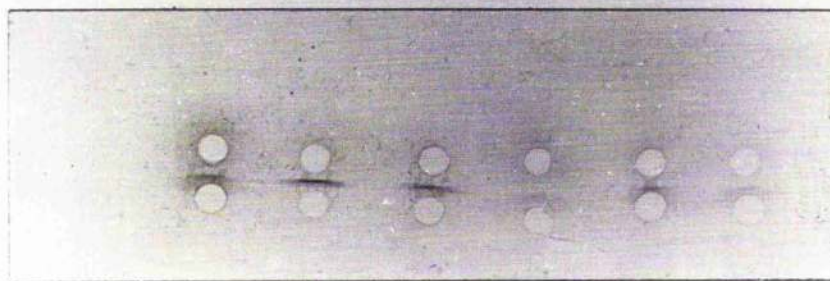
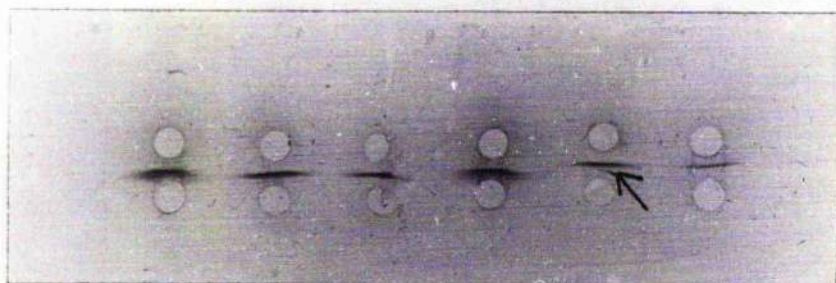


Plate 4 : Determination of the range of concentrations of pooled
day 61 convalescent sera and of goat anti-mouse IgA
used to precipitate IgA

Anti IgA ^a dilutions	$\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$
	0	0	0	0	0	0
	0	0	0	0	0	0

C.S.^b dilutions 1/5 1/10

Anti IgA dilutions	$\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$
	0	0	0	0	0	0
	0	0	0	0	0	0

C.S. dilutions 1/20 1/40

a Anti IgA = Goat anti- mouse IgA

b C.S. = pooled day 61 convalescent sera

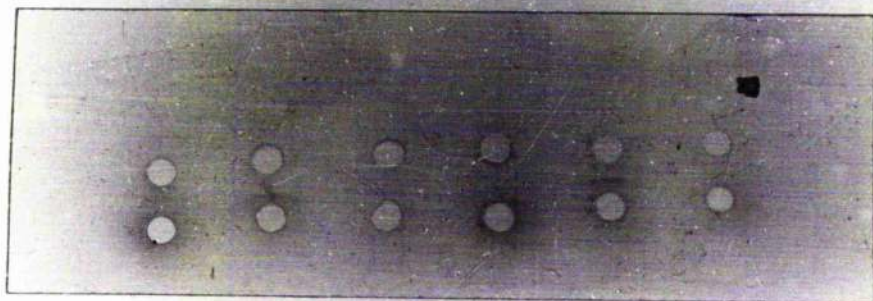
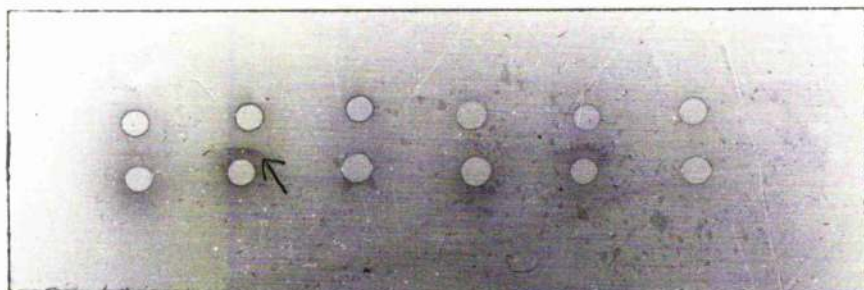


Plate 5 : Determination of the range of concentrations of 10x
concentrated pooled day 48 convalescent TBW and of goat
anti-mouse IgA used to precipitate IgA

Anti IgA ^a	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$
	0	0	0	0	0	0
	0	0	0	0	0	0

C. TBW^b Neat 1/2

Anti IgA	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$
	0	0	0	0	0	0
	0	0	0	0	0	0

C. TBW 1/5 1/10

a Anti IgA = Goat anti-mouse IgA

b C. TBW = 10x concentrated pooled day 48 convalescent TBW

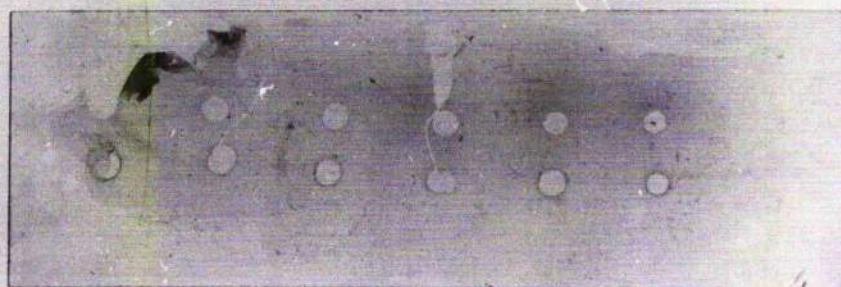
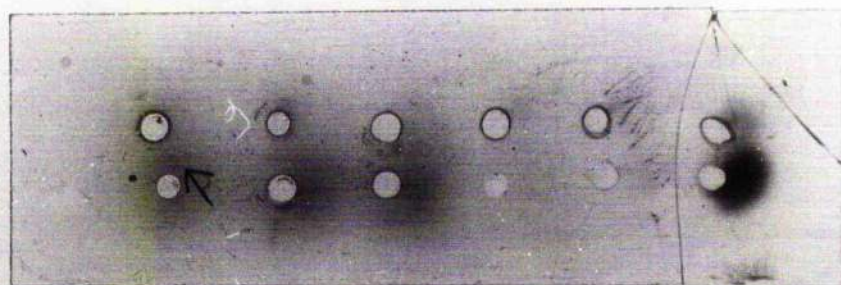


Table 6 : Summary of dilutions used to determine optimal ratios of convalescent sample
and goat anti-mouse immunoglobulin

Convalescent sample (Dilution)	Goat anti-mouse immunoglobulin (Dilution range)	Optimal dilutions for precipitation procedure (Optimal proportions)
Pooled day 61 convalescent serum (1/50)	Goat anti-mouse IgG (1/10 - 1/320)	1/10 : 1/50 (5 : 1)
Pooled day 61 convalescent serum (1/5)	Goat anti-mouse IgA (1/2 - 1/64)	1/4 : 1/5 (5 : 4)
10x concentrated pooled day 48 convalescent TBW (1/10)	Goat anti-mouse IgG (1/2 - 1/64)	1/5 : 1/10 (2 : 1)
10x concentrated pooled day 48 convalescent TBW (1/1) ^a	Goat anti-mouse IgA (1/1 - 1/32)	1/1 : 1/1 (1 : 1)

^a 1/1 = undiluted

mixtures at 37°C for 1 h. The mixtures were then centrifuged at 10,000 rpm for 2.5 min in the microfuge. The supernates were removed and kept at 4°C.

Immunodiffusion of these supernates was next done against wells containing the corresponding antigen and antibody. Glass slides (10 x 50 mm) with 5 ml of barbitone agar were prepared as previously described. A row of 3 mm wells were cut with 1 cm between each. Two troughs were cut with 1 cm between each, i.e. the two troughs were cut 0.5 cm from either side of the wells. One trough was filled with pooled convalescent sera and the other with goat anti-mouse sera. After 18 h at room temperature the slides were washed, stained, dried and inspected to determine which supernate failed to precipitate with either antigen or antibody. This was taken as the optimum proportion mixture. Appendix 13, 14, 15 and 16 contain the plates of each determination of optimal dilutions for the 4 groups given on Table 6.

4.5 Absorption of pooled sera with Phase I and Phase IV *B. pertussis*

The dose of Phase I *B. pertussis* 18-323 which removed all Phase I agglutinins from pooled day 48 convalescent serum was determined. Doses of 5, 10, 25, 50 and 100 o.u. ml (Wardlaw, 1980) of 24 h BG grown *B. pertussis* 18-323 were suspended in 1 ml saline and centrifuged for 2.5 min at 10,000 rpm in a microfuge (Beckman, High Wycombe, Bucks.). These pellets of live bacteria were resuspended thoroughly in 0.5 ml pooled day 48 convalescent serum to give final concentration of 10, 20, 50, 100, 200 o.u. *B. pertussis*. The

mixtures were left for 18 h at 4°C and microfuged again for 2.5 min at 10,000 rpm and the supernates removed. The concentration of bacteria which removed all B. pertussis Phase I agglutinins was found to be 200 o.u. and this was termed the Standard Absorbing Concentration (S.A.C.).

Aliquots of 0.5 ml of pooled day 48 convalescent sera were absorbed with 1 S.A.C. or 5 S.A.C. Phase I 18-323 or the same concentration of B. pertussis Phase IV D30042. Pooled normal serum was absorbed with 1 S.A.C. B. pertussis Phase I. All of the above absorptions were done with live organisms and the final treated sera were diluted to 1/10 in saline and millipore filtered before use.

4.6 Bacterial agglutination

A modification of the method of Preston (1970) was used. Saline (0.02 ml) was added to all wells except the first in each row in a Cooke Microtitre Tray (Sterilin Ltd., Sussex). Sample (0.02 ml) was added to the first 2 wells and doubling dilutions of the sample made, starting at the second well and using an automatic pipette with a fresh tip for each transfer. To each well, 0.02 ml of 30 o.u. live B. pertussis suspension prepared from 24 h growth on a BG plate was added.

Duplicate controls were prepared of (a) 0.02 ml of saline and 0.02 ml of 30 o.u. B. pertussis and (b) 0.02 ml of saline and 0.02 ml of serum or TBW. Microtitre trays were then secured into a Gallenkamp orbital shaker for 5 min at 180 rpm to ensure thorough mixing. The trays were incubated for 30 min at 56°C, removed and results read immediately and 2 h later. The agglutinin titre was the

reciprocal of the last dilution showing definite clumping visible to the naked eye with the tray illuminated obliquely from below.

4.7 Anti-F-HA

This antibody was assayed by haemagglutination inhibition as follows: Saline (0.05 ml) was added to each well except the first well in each row of a Cooke microtitre tray. Sample of serum or TBW being tested for antibody (0.05 ml) was added to the first two wells in each row and doubling dilutions made starting at the second well. Eight haemagglutinating units of culture supernate of B. pertussis strain 77/18319 grown in a modified Cohen and Wheeler Medium were added to each well and trays left at room temperature for 30 min. To each well was then added 0.05 ml of 2% Horse RBC and the trays incubated for 1-2 h at 37°C. The reciprocal of the highest dilution of sample to show complete inhibition of haemagglutination was recorded as the haemagglutination inhibition titre (HAI titre).

4.8 Anti LPS

PBS (0.05 ml) was added to all wells except the first in each row in a Cooke Microtitre Tray (Sterilin Ltd., Sussex). Sample (0.05 ml) was added to the first 2 wells and doubling dilutions of the sample were made starting at the second well using an automatic pipette with a clean tip for each transfer. To each well, 0.05 ml of 2% HRBC previously coated with LPS (preparation in Appendix 5) was added.

Duplicate controls were prepared consisting of (a) 0.05 ml of

veronal buffer and 0.05 ml of sample, (b) 0.05 ml of PBS buffer and 0.05 ml of 2% coated HRBC, (c) 0.05 ml of PBS buffer and 0.05 ml of 2% uncoated HRBC and (d) 0.05 ml of sample and 0.05% of untreated HRBC.

Mixtures were shaken and results read after 18 h at room temperature. The reciprocal of the highest dilution showing full agglutination of the LPS-coated HRBC was taken as the agglutination titre.

4.9 Anti-LPF in the convalescent mouse

B. pertussis-infected mice at various times after initiation of a pulmonary infection, and normal mice, all at approximately the same age, were injected intraperitoneally with 0.5 ml of three graded doses of Glasgow Standard Pertussis Vaccine (as in 4.1). Five days after inoculation, blood was removed for leucocyte count (as in 2.6). After the mice had recovered from anaesthesia, they were replaced in their cage and their histamine sensitivity was tested the following day.

4.10 Anti-HSF

4.10.1 In convalescent serum and TBW

Pooled sera and TBW were mixed with equal volumes containing either 10 or 20 HSD₅₀ of Glasgow Standard Pertussis Vaccine suspended in saline; after thorough mixing the samples were held for 18 h at 4°C. Six to seven wk old HAM 1/CR mice of either sex were injected intraperitoneally in groups of 7 or 14 with 0.2 ml of mixture thereby giving each mouse 1 or 2 HSD₅₀ (in the absence of neutralisation by antibody). Groups of 7 mice of 6-7 wk were injected intraperitoneally

with 0.2 ml of graded doses of vaccine sample alone to check the number of HSD₅₀ being used. Additional control mice were injected with saline. Five days later histamine sensitivity was measured as in 2.7.

4.10.2 In the convalescent mouse

B. pertussis infected mice at various stages of convalescence from pulmonary infection as in 4.9 with normal mice of the same ages as controls were examined for histamine sensitivity as in 2.7.

4.11 Anti-HLT

Anti-HLT was assayed by neutralization of haemorrhagic activity of B. pertussis cell-lysates in 4-day old suckling mice. For maximum sensitivity, the tests were done by mixing the HLT-containing lysate with an equal volume of serum or TBW so that the final mixture contained 1 standard haemorrhagic dose (a dose which produced significant haemorrhage) in 0.05 ml. After 18 h at 4°C, each mixture was injected into a group of 2 or 3 suckling mice which were inspected 24 h later for haemorrhagic reaction.

4.12 Bactericidal activity

A dilution of 10^4 organisms ml⁻¹ was made from 24 h BG growth of B. pertussis 18-323 suspended in CAA and 0.5 ml was added to 0.5 ml of the serum or TBW samples. CAA (0.5 ml) was used as control to determine the cfu of the organism added. Mixtures were shaken and incubated at 37°C for 90 min when samples were removed and duplicate

0.025 ml portions spread on BG plates. These were incubated at 35°C for 5 days and the mean cfu were determined.

4.13 Crossed-over immunoelectrophoresis

The composition of reagents is given in Appendix 4. The method of Culliford (1964) was followed. Clean glass slides (75 mm x 50 mm) were placed on a levelling tray and 12 ml of molten 0.75% barbitone agar were spread evenly onto the slides using a pipette. The agar was allowed to solidify and left for 20 min at 4°C. Wells of 3 mm were cut in paired rows with 1 cm between the adjacent wells and 0.5 cm between the rows. Within each pair of rows samples were dispensed in the row placed nearest the anode and antigen preparation in the row opposite and put nearest the cathode on the electrophoresis chamber (Shandon type). Antigens, Phase I 18-323, Phase IV D30042 and C Mode 18-334 were prepared by scraping 72 h growth on BG plate into CAA to give a thick suspension. They were sonicated (Ultrasonic Disintegrator, Measuring and Scientific Equipment Ltd., London) for 6 x 20 sec until the suspension had become opaque. Wells were filled using an automatic pipette.

The slides were placed in the electrophoresis chamber with the antigen preparation of each paired row near the cathode and electrophoresed for 30 min at 100 volts (Vokam power pack).

Slides were left in barbitone buffer and left overnight to remove unreacted protein. They were washed twice in distilled water and stained for 10 min in amino black saturated solution (E. Gurr Ltd., Michrome Ltd., High Wycombe, Bucks). The gels were destained

in 2% acetic acid for 24 h. The slide was then placed in 2% acetic acid/1% glycerol to prevent the dried gel flaking off the slide. Slides were dried with a thick pad of tissues with a weight on top for approximately 30 min. Finally the slides were completely dried with a blow-drier.

4.14 Quantitative immunoelectrophoresis

This was done by the method of Hertz et al (1979) in which the test samples were incorporated into the intermediate gel ($40 \mu\text{l cm}^{-2}$) using B. pertussis 18-334 sonicate reference antigen and rabbit antiserum (Hertz antiserum as in 4.2) in the back gel.

Antigen preparation was as follows: Phase I B. pertussis 18-334 grown on BG medium at 35°C for 72 h was suspended in modified Hornibrook medium containing 5 g NaCl l^{-1} to give X mode cells. The cells were incubated at 35°C for 72 h, then sonicated for $3 \times 45 \text{ sec}$ exposures, ice-cooled, at $20,000 \text{ Hz/sec}$ using a Rapidis 300, 19 mm probe with a 9.5 mm tip. The sonicate was then centrifuged at $48,000 \text{ g}$ for 60 min at 4°C and the supernatant was stored at -30°C . The colloid concentration of supernatant used was 12.5 g l^{-1} .

Rabbit antisera to the sonicate was prepared for the back gel as follows: Rabbits were immunized intradermally with sonicate in Freund's Incomplete Adjuvant, each animal receiving $100 \mu\text{l}$ of antigen per immunization. All rabbits were immunized for at least 6 months.

Immunoelectrophoretic analysis of test samples was performed on $5 \times 5 \text{ cm}$ glass plates using 1% agarose (Indubiose A 37) in Tris-

barbital buffer, pH = 8.6 ionic strength = 0.02.

First dimension electrophoresis of 2 μ l of antigen were run for 30 min at 12°C 10 volts cm^{-2} , second dimension electrophoresis for 18 h, 2V cm^{-2} . Test samples were in the intermediate gel and rabbit antisonicate (20 μ l) in the second gel. Coomassie Brilliant Blue - R was used to stain the precipitates.

4.15 Sublethal intranasal passive protection activity

Pooled serum and TBW were titrated for ability to protect 3-4 wk old mice from SLIN challenge with approximately 1.25×10^5 cfu of 24 h BG grown B. pertussis 18;323 (P). Serial dilutions of samples were made and equal volumes of each mixed with the challenge suspension (SLIN/mix) (North, 1946). Controls included the same dose of cells mixed with 1% CAA. The mixtures were allowed to stand for 15 min and then administered to the mice in not more than 75 min thereafter. Each was instilled in 2 x 0.025 ml drops into ether-anaesthetized mice as in 2.2. To minimize bias mice were distributed between cages so that each cage contained animals representing each type of treatment.

Pooled day 48 convalescent serum (at 1/20 dilution) and TBW (at 1/2 dilution) were also intranasally instilled in 2 x 0.025 ml drops to each mouse at intervals of 48, 24 or 6 h before the mice were challenged with approximately 1.25×10^5 cfu each. This was to see if passive protection could be achieved by administering the serum or TBW before the challenge.

The mice were inspected daily for 14 d on which day they were autopsied as in 2.8. The difference between the results from control

and test groups were tested for their statistical significance by the 2 x 2 contingency tables of Finney et al (1963).

4.16 Intracerebral passive protective activity

Pooled serum and TBW were tested for their ability to protect 3-4 wk old mice against a lethal IC challenge of 3×10^4 B. pertussis. Equal volumes of pooled serum and TBW were mixed with challenge suspension (IC/mix) and intracerebrally administered as in 2.3 (the "IC/mix" test of Dolby and Standfast, 1958). The mixtures stood for 20 min before inoculation, but not longer than 90 min before being inoculated. Mice were caged such that each cage contained animals representing each type of treatment.

Mouse deaths were recorded for 14 d after the inoculation. Mice dying within 3 d of challenge were excluded from the final count, and not considered as "infection deaths". Mice moribund on day 14 were counted as dead. Difference between the results from control and test groups were tested for their statistical significance by the 2 x 2 contingency tables of Finney et al (1963).

SECTION V STATISTICAL ANALYSIS

5.1 Estimation of parameters

Counts of B. pertussis in the lungs of infected mice were summarized as the geometric mean as done by Dolby et al (1961). Variability was expressed as the anti-logarithm of the standard deviation of the logarithm of the counts.

Leucocyte counts in the blood of mice given pertussis vaccine or saline were summarized as the geometric mean and 95% CL since such counts are approximately log-normally distributed (Idigbe, 1979).

Lung pathology were summarized as the arithmetic mean and 95% CL.

5.2 Measure of association between two normal variables

The product-moment correlation coefficient (Campbell, 1974) was used to measure the association between (a) cfu in lungs and pathology score of those lungs, (b) infecting dose of B. pertussis and mortality rate and (c) reciprocal agglutinin titre in sera to 18-323 and 18-334.

5.3 Assessment of significance of differences between experimental groups

5.3.1 The t-test

Results for the neutralization of LPF at each graded dose of vaccine in normal and infected mice were analysed by the t-test on the data expressed in logarithms to the base 10.

5.3.2 Mann-Whitney U-test

The Mann-Whitney U-test was employed to compare certain types of data where parametric tests seemed inappropriate, eg scores from lung pathology and lung culture from B. pertussis infected mice. The procedure was as described by Campbell (1974).

5.3.3 Use of 2 x 2 contingency tables

For the comparison of data in the form of proportions (eg mortality ratios), 2 x 2 contingency tables were consulted (Finney et al, 1963). These were used to test the significance of differences in response for groups of mice given different treatments, eg passive protection afforded to groups of mice against an intracerebral or intranasal challenge with B. pertussis.

5.4 Estimation of PD₅₀ in animal challenge experiments

For analysis of dose-response relationships (eg passive protection tests) where the response variable was a binomial proportion, the probit transformation was used. Data were processed by the probit method (Finney, 1971) using a computer programme developed in the Department of Epidemiology and Biometrics, School of Hygiene, University of Toronto, Canada. The University of Glasgow ICL2976 computer was used to run this programme which yielded PD₅₀ values and the relative potencies compared with a standard, with 95% confidence limits.

RESULTS

SECTION I SOME CHARACTERISTICS OF THE MURINE SUBLETHAL PULMONARY
INFECTION WITH B. PERTUSSIS

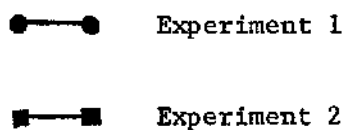
The main purpose of the investigation described in this thesis was to characterize the nature of the immune response of mice given a sublethal pulmonary infection with B. pertussis. Of particular interest was the comparison of acute-phase and convalescent sera and TBW, and it was therefore necessary to define the infection peak in the lungs of the experimental animals. Moreover, for monitoring passive protective activity of convalescent serum and TBW, it was necessary to have already determined the peak of infection so that observations in test animals could be made at that time.

1.1 Time course and infection peak

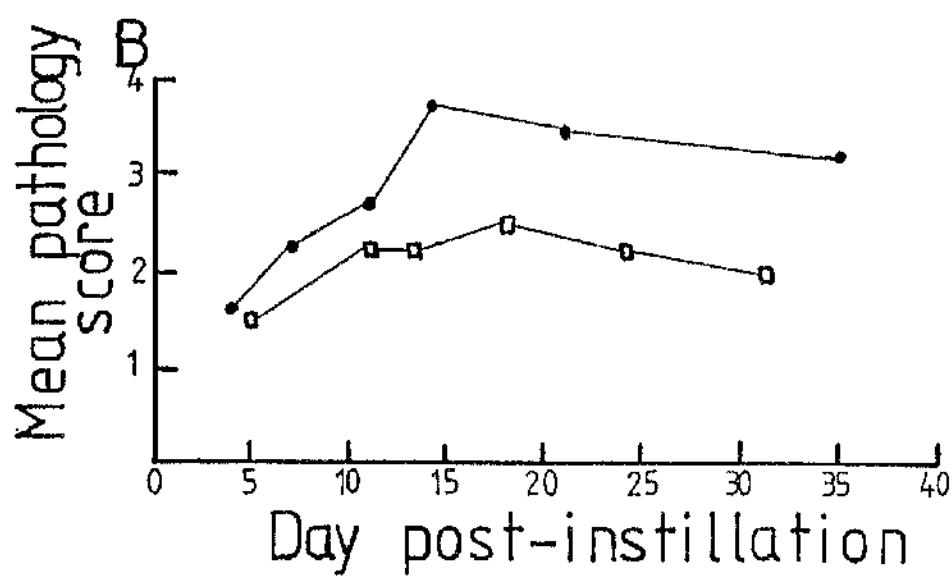
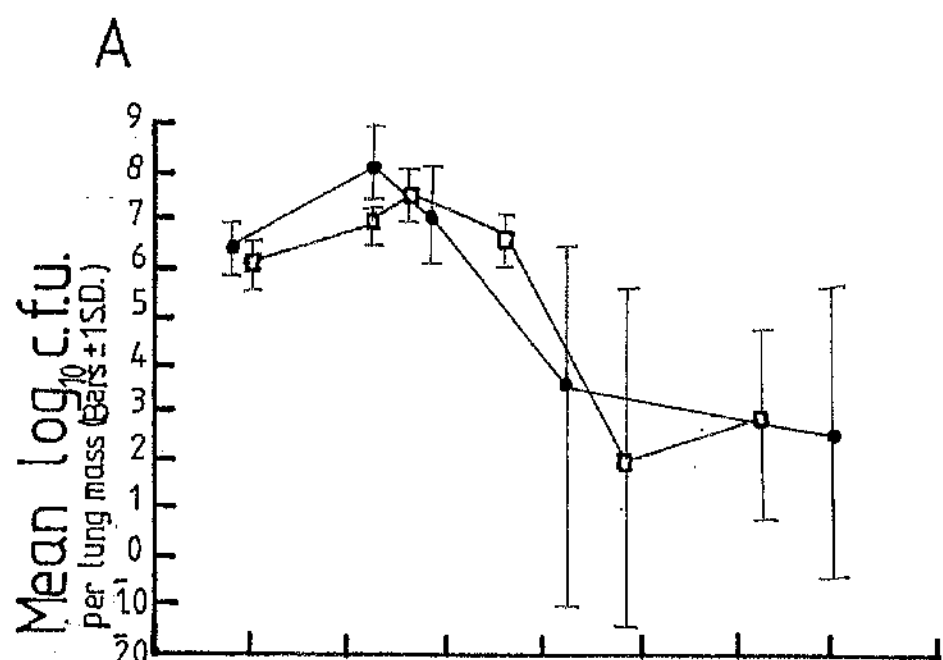
In two independent experiments, pulmonary infection was established in 3-4 week old HAM 1/CR mice of both sexes by the IN instillation of 4×10^5 cfu (Expt 1) and 1×10^5 cfu (Expt 2) of B. pertussis strain no. 18-323 (P). At suitable intervals after infection 3 or 4 mice were sacrificed and the thorax of each animal opened with sterile precautions. The lungs were examined for degree of consolidation and a pathology score assigned on a 0 to 4 scale (Fig 3b). The lungs were then excised, homogenized in sterile CAA and serially diluted for determination of cfu by surface colony counts on BG medium.

Figure 3a records the mean cfu of B. pertussis in the lungs during the 31 days (Expt 1) or 35 days (Expt 2) of observation. At

Figure 3 : Development of murine pulmonary infections with B. pertussis 18-323 (P) from an initial challenge of either 4×10^5 (Experiment 1) or 1×10^5 (Experiment 2) cfu as determined by the mean^a cfu (A) and pathology score (B) in the lungs. The error bars of A represent 1 SD.



a Each point is the mean of 3 or 4 mouse lungs.



day 4 and 5, the earliest post-instillation observation day for both experiments, the mean count was just over 10^6 cfu per individual mouse lung mass. Thereafter the count increased further, reaching a peak of 10^8 on day 11 in Expt 1, where the larger challenge had been given and 10^7 on day 14 in Expt 2. Subsequently, the counts declined at a rate apparently not dissimilar from that at which they had increased, but final clearance of all live bacteria had not been achieved even at 31-35 days. For example, the 31 day mice in Expt 2 yielded 4.5×10^4 , 2.5×10^3 , 7.5×10^3 and $< 10^2$ cfu from individual animals, and the 35 day mice from Expt 1 yielded 9.8×10^5 , 3.4×10^4 , $< 10^2$ and $< 10^2$ cfu. The limit of detection was 10^2 cfu/lung mass.

In other experiments where pooled TBW was collected at 48 days post-instillation and plated onto BG agar, no growth was obtained showing that infection although persisting for 35 days had disappeared by 48 days. For purposes of sacrificing mice at the peak of infection, 14 days post-instillation was taken as the standard time.

The parallelism of development of lung cfu and lung pathology scores was investigated by the comparison afforded in Fig 3b. Significant consolidation in the lungs was already visible in Experiment 2 by day 4 (mean score 1.5) and in Experiment 1 by day 5 (mean score 1.6). Thereafter, the pathology scores increased, with the larger infecting dose (4×10^5) of Expt 1 yielding a higher mean maximum score of 3.75 than the 2.5 of Expt 2. Fig 3b shows that the pathology score reached plateaus rather than sharp peaks and that this occurred between day 12 and 17. The difference in plateau scores in the two expts was significantly different ($P < 5\%$) by the Mann-Whitney U-test. By day 35 in experiment 1, the mean pathology

score was still at 3.25 (out of a maximum of 4.0) although the mean count at this time was nearly 10^6 times lower than it had been at the peak. It was apparent therefore, that significant pulmonary consolidation persisted long after the infection peak had passed and long after the main burden of bacteria had been eliminated.

In other experiments, consolidation was found on day 42 after instillation but had disappeared by day 49. For details of pathology scores and bacterial counts in the individual mice in Fig 3a and 3b see Appendix b.

1.2 Correlation between cfu and pathology score

In view of certain similarities in Figure 3 between mean changes in bacterial count and in lung pathology, it was decided to examine individual animals for the degree of correlation between these two variables. Where cfu were $< 10^2$ an arbitrary value of 0 cfu was allocated and where cfu were uncountable because of confluent growth the value of 10^{10} was allocated. From the two experiments, a total of 36 paired observations was available and these were plotted as a correlation diagram, which is not provided here but which showed no significant correlation between the two variables. The product-moment correlation coefficient 0.095 was statistically not significant ($P > .5\%$). Nevertheless it was noted that there appeared to be some correlation between pathology score and cfu in those mice which had been sacrificed before and including the infection peaks. Therefore the data were divided into two approximately equal groups, one consisting of results that had been gathered up to day 11 in expt 1 and day 13 in expt 2, and those gathered thereafter.

Two separate correlation diagrams were then plotted and are presented as Fig 4a and 4b. The product moment correlation coefficient of Fig 4a is 0.52 which is just a significant correlation (P at 5% level ≈ 0.48) between pathology score and cfu during the time period before and including the infection peak. However after the infection peak the correlation coefficient in Fig 4b is 0.38 (P at 5% ≈ 0.41) and thus does not quite reach the conventional level for a significant correlation score and cfu after infection. The main point to emerge is that significant correlation exists between pathology score and cfu while the organism is actively multiplying during the initial stage of infection (Fig 3a). However the correlation weakens as the infection passes its peak and the infection burden is in decline (Fig 3b).

1.3 Mortality rate in sublethal intranasal infections

To discuss mortality rate in B. pertussis infections which are described as "sublethal" may appear paradoxical, although there is precedence for this in the literature (Pittman, 1951). For purposes of the present investigations, "sublethal" is taken to mean "a mortality rate preferably below about 30% and certainly less than 50%".

The record of mortality observed in the large groups of mice that were infected so as to yield convalescent serum and TBW is set out in Table 7. In the course of the investigations extending over 3 years, a total of 924 mice was used in 8 separate experiments and the overall mortality rate was 22%. However this conceals some notable variations in the source of the B. pertussis strain and in the sampling time. No mortality occurred in the normal mice.

- Figure 4.
- A. Correlation between lung pathology score and c.f.u. before and including the infection peak.
 - B. Correlation between lung pathology score and c.f.u. after infection peak.

Table 7 : Mortality rate in the infection experiments used to raise pooled sera and TBW

Expt No.	Source ^a of Strain 18-323	Day post-instillation when experiment terminated	No. of mice		c.f.u. of infecting dose (x 10 ⁵)	% Mortality
			Initially infected	No. of survivors		
1	M.P.	42	120	114	1.9	5
2	F.S.	49	130	93	2.8	28.4
3	F.S.	15	90	90	2.3	0
4	M.P.	48	118	91	ND ^b	23
5	M.P.	34	119	76	1.7	36
6	M.P.	40	40	30	1.4	25
		47	40	30	1.4	25
7	M.P.	61	140	103	1.8	26
8	M.P.	61	127	96	ND	24

^a M.P. = Dr. M. Pittman, Bethesda, Maryland

F.S. = Dr. F. Sheffield, N.I.B.S.C., London

^b ND = Not determined.

Two different sources of strain 18-323 were used. The strain supplied by Dr. F. Sheffield (National Institute of Biological Standardization and Control, London) had a high IN virulence when first received and at this time it was used in expt 2 and 3. In both these experiments, significant pathological changes were recorded and B. pertussis was recovered from the lungs on day 14 after instillation. Subsequently, however, a sharp drop in virulence of this strain occurred. Therefore strain 18-323 supplied by Dr. Pittman (Bureau of Biologics, Bethesda) was used in all the other IN infection experiments described in this thesis. The virulence of this latter strain stayed at a high level. (Note however that the converse is true while in the IC infections with the strain supplied by Dr. F. Sheffield maintaining its virulence and Dr. Pittman's strain diminishing in IC virulence.)

In expt 3, pooled day 15 acute phase serum and TBW samples were obtained before any deaths due to infection had occurred. Nevertheless, there was evidence that this had been an infection with the expected level of virulence since there was abundant oozing of fluid from the incised trachea at the time of cannulation to obtain TBW. This suggested that the lungs were significantly congested. In experiments 1, 2 and 4 to 8 the mortality rate was between 23-36% except in expt 1 which had a low mortality rate of 5% for no apparent reason.

Records of the day of death were kept for experiments 4 and 5 which were terminated day 48 and day 34 post-instillation respectively (Fig 5). In experiment 4, deaths started around day 16 post-instillation with no more being recorded after day 41. In experiment 5, deaths

Figure 5. Daily record of deaths in the infection experiments 4 and 5 which were used to provide pooled sera and TBW from day 48 and day 34 post-instillation. Each point represents one mouse.

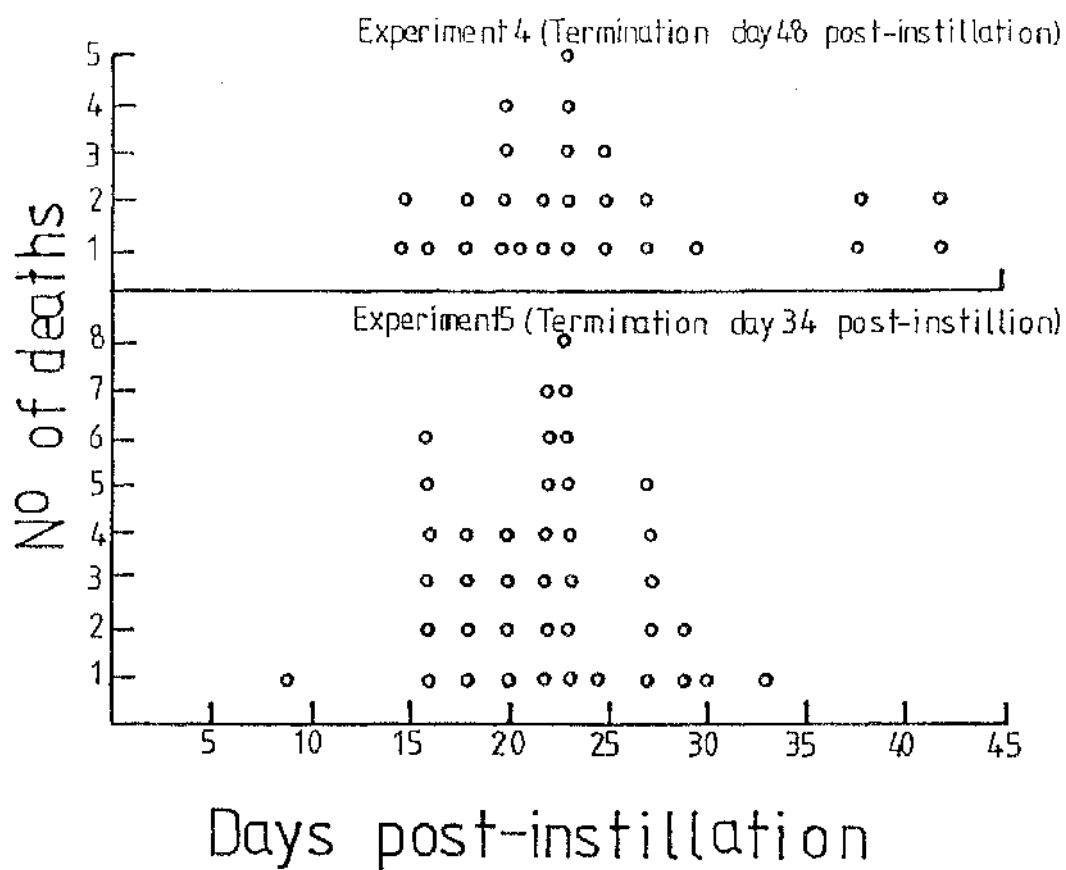
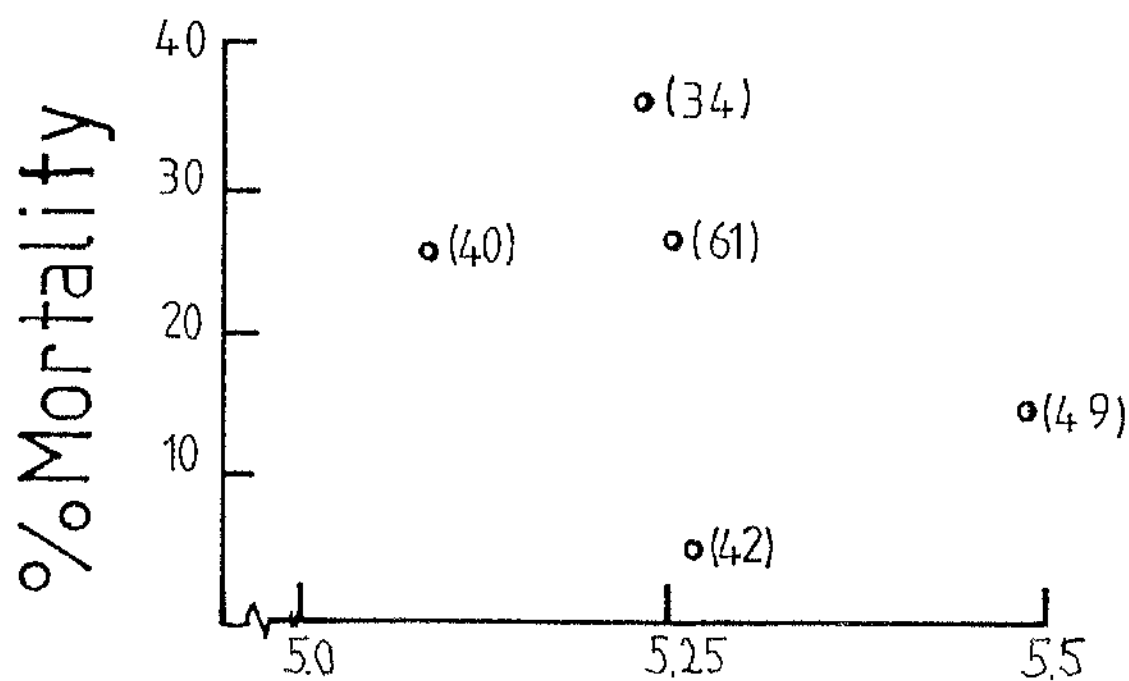


Figure 6. Correlation between the cfu in the infecting dose and the subsequent % mortality of the recipient mice. Numbers in parenthesis are the days on which the experiment was terminated.



Log₁₀ c.f.u. of
infecting dose

commenced on day 9 post-instillation and continued until termination day 34. In both experiments the daily peak no of deaths were on day 23 and deaths were recorded until day 42 of experiment 4 and day 33, the day before termination, of experiment 3.

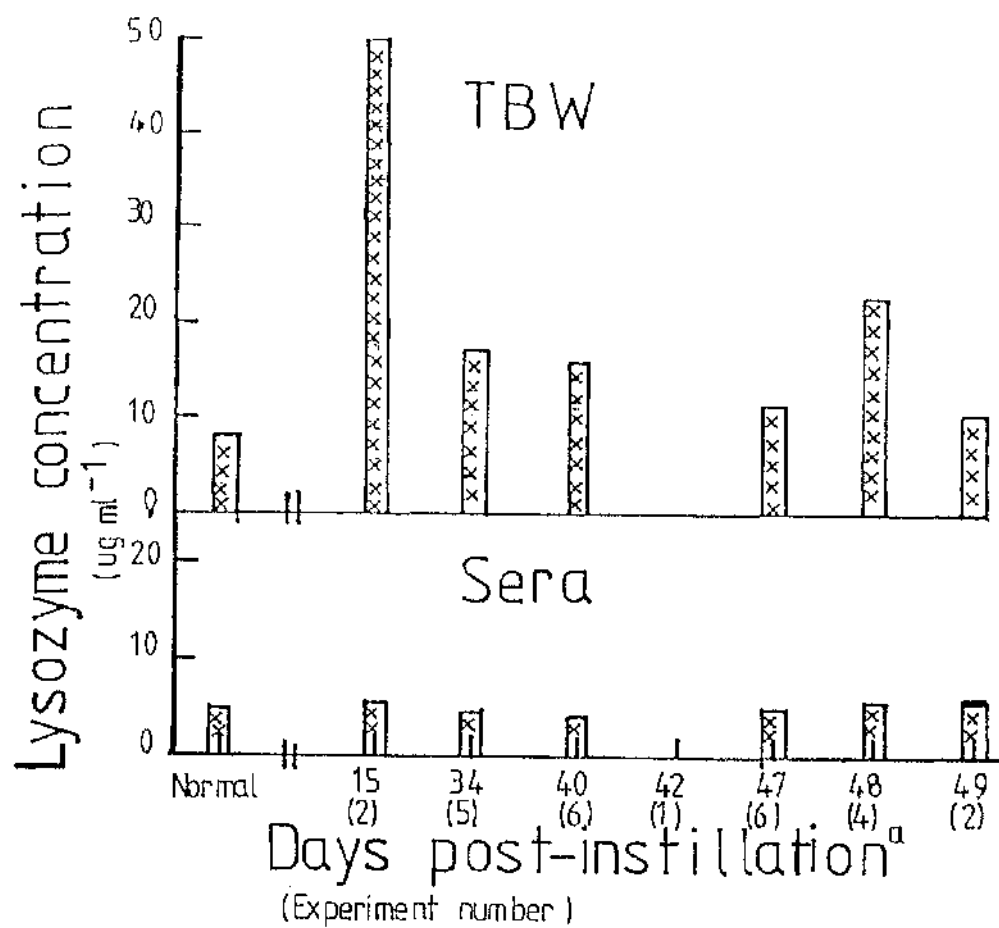
The question of correlation between the mortality rate and the cfu in the infecting dose is investigated in Fig 6. In short there was no correlation (product-moment correlation coefficient = 0.29). But it should be noted that the 2-fold dose range was very small compared with what is customary in a bacterial virulence titration.

1.4 Lysozyme concentrations in pooled TBW and serum

Lysozyme determinations were made on pooled TBW from infected mice in experiments 1-8 as a measure of the non-specific inflammatory response to infection (Fig 7). Normal, uninfected animals of the same age were used as controls. The latter animals had a mean lysozyme level of $8 \mu\text{g ml}^{-1}$. However lysozyme concentrations of $50 \mu\text{g ml}^{-1}$ in pooled day 15 acute phase TBW was 6 times that of normal pooled TBW. This coincided with the presence of heavy congestion in the lungs at this time. Lysozyme concentrations had returned to normal levels by day 61 post-instillation with pooled day 61 convalescent TBW having $8 \mu\text{g ml}^{-1}$ of lysozyme.

The pooled TBW gave no idea of the variation in lysozyme concentrations between individual animals. This question was explored by sampling 10 individual TBW from normal mice at 3 wk and 12 wk old and mice which had been infected with 1.0×10^5 cfu B. pertussis at 3 wk of age and then sampled 14 day and 61 day post-instillation

Figure 7. Lysozyme concentrations in pooled TBW and sera from normal and B. pertussis infected mice at different times post-instillation.



^a Note this is not a linear scale

Table 8 : Lysozyme concentrations in TBW from individual normal mice at different ages and from individual *B. pertussis* infected mice at different stages post-instillation

Type of mouse	Age of mouse (weeks)	Day post-instillation when TBW obtained	Lysozyme concentration ($\mu\text{g}/\text{ml}^{-1}$)										Mean (95% C.I.)
			Individual mice										
Normal	3	-	6.3	6.7	7.9	6.0	4.8	9.9	6.7	7.9	8.9	-	7.2 (6.0-8.4)
Normal	12	-	7.9	5.7	4.8	4.8	7.5	8.9	5.7	3.2	6.3	6.7	6.15 (4.9-7.3)
Infected	5	14	10.3	6.1	25.0	22.1	47.8	30.0	35.0				25 (15.7-34.8)
Infected	12	61	5.8	5.5	5.7	7.3	9.7	8.9	6.3	5.7	14.0	11.8	8.07 (5.9-10.2)

normal
 Table 9 : Lysozyme concentrations in homogenized washed lungs of individual mice at different ages and individual *B. pertussis* infected mice at different stages post-instillation

Source of lung	Age of mouse (weeks)	Day post-instillation when lungs obtained	Lysozyme concentrations ($\mu\text{g}/\text{mouse lung mass}$)		
			Individual mice		Mean (\pm 95% C.L.)
Normal	3	-	22.5	28.5 31.5 -	27.5 (16.2-39.0)
Normal	12	-	14.5	14.5 21.5 22.5	18.2 (11.6-25.2)
Infected	5	14	132.0	26.0 55.0 41	63.6 (42-85.2)
Infected	12	61	21.5	15.0 17.0 30.0	21 (10.5-31.5)

(Table 8). The range of lysozyme concentrations were similar in normal mice at 3 wk and 12 wk of age and also at day 61 post-instillation of infected mice. The mean lysozyme concentrations for these groups were 7.2, 6.1 and 8.1 $\mu\text{g ml}^{-1}$ respectively. Lysozyme concentrations in individual TBW did not alter with age and in individual infected mice at day 61 post-instillation had nearly all returned to normal levels.

Individual TBW sampled on day 14 post-instillation (infection peak) had a wider range of lysozyme concentrations and the mean value (25 $\mu\text{g ml}^{-1}$), lower than found in pooled day 15 acute phase TBW, was significantly elevated from either the normal or mice at day 61 post-instillation of infection.

Individual lung homogenates from the normal mice at 3 and 12 wk and infected mice 61 days post-instillation also had a similar range with a mean lysozyme content of 27.5, 18.25 and 21 $\mu\text{g}/\text{mouse lung mass}$ respectively (Table 9). Therefore 1/4 - 1/5 of the lysozyme content of the lungs from either normal or mice 61 days after infection was in the lung washings. Individual lung homogenates from mice at day 15 of a pulmonary challenge had a mean value of 63.6 $\mu\text{g}/\text{mouse lung mass}$, ie nearly 1/3 of the lysozyme content, slightly more than in normal or convalescent mice was recovered by lavage from mice at the infection peak. A slightly higher proportion of lysozyme is in the secretions at infection peak than in the tissues.

1.5 Protein concentration of pooled TBW

Protein determinations were made on pooled TBW from normal and

Table 10 : Protein concentrations of pooled TBW from normal and
B. pertussis infected mice obtained at different times
post-instillation

Source of TBW	Day post- instillation when TBW obtained	No. of mice contributing to TBW pool	Expt No.	Protein concentration mg/ml
Normal	-	90	1	0.46)
Normal	-	80	2	0.25) m=0.36
Infected	15	90	2	1.5
Infected	42	114	1	0.93
Infected	49	93	2	0.38

B. pertussis infected mice in Expt 1 and 2, firstly to determine the protein ratio of TBW in relation to serum for immunological comparison, and secondly to see how protein concentration varied during infection (Table 10). With normal mice there was nearly a two fold difference in the protein level in TBW in experiments 1 and 2. This was not due to any technical error in the protein estimation because the two samples were run strictly in parallel. Pooled day 15 acute phase TBW showed a 4-fold increase over the mean normal protein concentration and coincided with the massive congestion in the lungs on this day. Pooled day 42 and 49 convalescent TBW had protein concentrations which although elevated from that of pooled normal TBW seemed to be returning to normal. The ratio of protein concentration between serum in relation to pooled normal TBW was 100:1, but was reduced to 50:1 in pooled day 42 and day 49 convalescent TBW.

SECTION II IMMUNOLOGICAL INVESTIGATIONS OF POOLED ACUTE PHASE AND CONVALESCENT SERA AND TBW

The immune response to the murine pulmonary infection with B. pertussis strain no 18-323 was determined by analysis of pooled convalescent sera and TBW by various in vitro and in vivo assays with pooled normal and acute phase samples included for comparison. The U.S. Standard Antipertussis Serum was used in all tests as a reference serum available to other investigators.

2.1 Agglutination Tests

Because agglutinins have been the most extensively studied

serological response of both mice and humans to B. pertussis infection, it was convenient to measure them at an early stage of the present investigation. It was also of interest to measure agglutinin not only against the 18-323 strain used for infection but also against other strains of B. pertussis. For this purpose strain 18-334, standard Phase I vaccine strain, was used both as X-mode and C-mode suspensions. Thus the pooled sera and TBW from infected mice were titrated for agglutinins against 18-323, 18-334 X-mode and C-mode. Normal pooled sera and TBW were also assayed, as was the U.S. Standard Antipertussis serum (Figure 8).

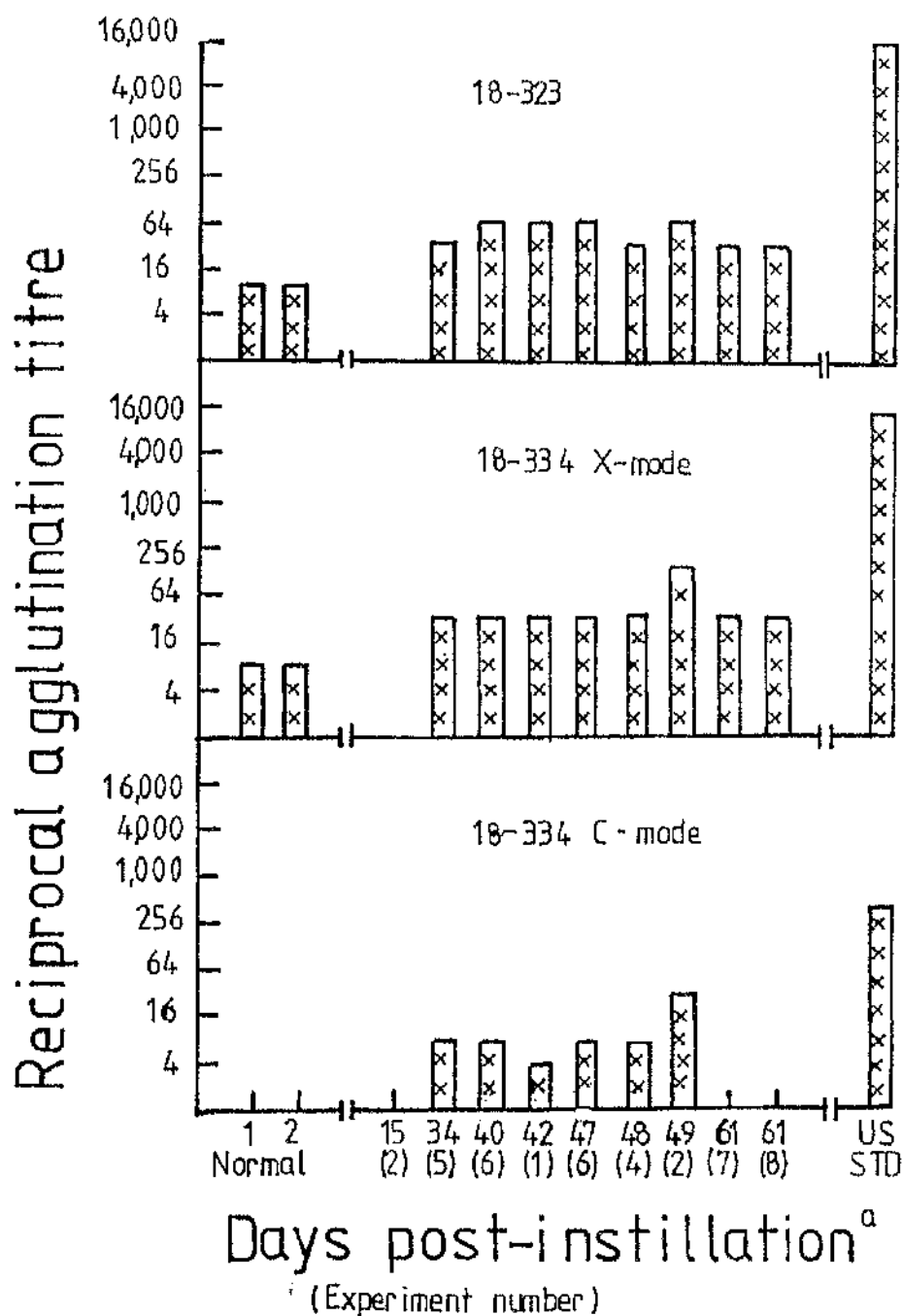
Taking the last-mentioned first, it had a reciprocal agglutination titre of 16,000 towards both X-mode strains 18-323 and 18-334 but only had a titre of 512 towards 18-334 C-mode.

Pooled convalescent day 34 serum had a reciprocal titre of 32 towards both of the X-mode strains and 8 towards 18-334 C-mode. This titre for X-mode organisms persisted unchanged in pooled day 61 convalescent serum. However agglutinins were not present at this stage towards 18-334 C-mode.

An unexpected result was that normal serum obtained from two different batches of mice both had a reciprocal titre of 8 towards the X-mode strains, as did pooled day 15 acute phase serum. However neither pooled normal or day 15 acute phase sera had any agglutinins to 18-334 C-mode. Neither pooled TBW from normal nor from infected mice contained demonstrable agglutinins.

The unexpected observation that pooled normal mouse serum contained agglutinins to B. pertussis and that the reciprocal agglutinin titre of infected mice was not increased greatly, led to a study of

Figure 8. Reciprocal agglutination titres of pooled sera from normal and B. pertussis infected mice at different times post-instillation. The sera were tested for agglutinins against 18-323 (P), 18-334 X-mode and 18-334 C-mode strains. The U.S. Standard Antipertussis Serum was used as a reference.



^a Note this is not a linear scale

sera from individual normal mice. Normal animals were bled at 3 wk and 12 wk of age and individual infected mice after 2 wk and 9 wk post-instillation. All these individual sera were titrated to determine the consistency of reciprocal agglutinin titre within each group (Table 11). Individual normal sera from both 3 wk and 12 wk old mice had a reciprocal agglutinin titre for both 18-323 and 18-334 ranging from 0 to 32. There was no significant difference in the reciprocal titre for the two strains of B. pertussis or between the two age groups of mice. There therefore appeared to be a low titre B. pertussis agglutinin in the serum of normal HAM 1/CR mice. Individual day 15 acute phase sera also had a reciprocal agglutinin titre for both 18-323 and 18-334 ranging from 0 to 32 with no significant difference in the reciprocal titre for the two strains of B. pertussis. It is noteworthy that infection did not seem to act as a booster stimulus in what might be presumed to be primed animals.

Twelve wk old mice at day 61 of infection had a significantly elevated agglutinin titre towards both the B. pertussis strains. However since paired sera samples were not obtained for individual mice, it is not known if each mouse had an increase in agglutinin titre after infection as a few infected mice gave sera with agglutinin titres within the normal range.

Possibilities of correlation between the reciprocal agglutination titres for the 2 strains of B. pertussis with sera from the 4 types of mice (as tabulated in Table 11) is investigated in Fig 9. The correlation coefficients of Fig 6a-d were 0.47, -0.03, 0.65 and 0.36, respectively, of which only the third mentioned was significant.

Table 11 : Reciprocal agglutination titres of individual sera from normal mice at different ages and individual *B. pertussis* infected mice at different stages post-instillation infection.
Strain 18-323 and 18-334 X mode were used as antigens in the agglutination tests

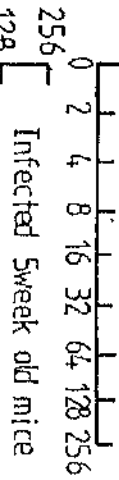
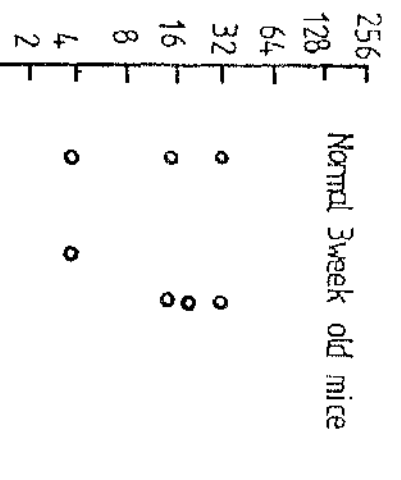
Source of Sera	Age of mouse (weeks)	Day post-instillation when TBW obtained	Agglutinating strain	Reciprocal agglutination titre											G.M. (\pm 95% C.L.)
				Individual mouse titres ^a											
Normal	3	-	18-323	32	32	4	32	32	0	16	4	4	4	N.T.	10.0 (3.7-27.4)
			18-334-X	16	16	32	32	16	4	4	16	4	4	N.T.	15.3 (8.0-29.6)
Normal	12	-	18-323	4	16	16	16	8	16	32	32	16	8	8	13.9 (8.8-21.9)
			18-334-X	16	16	32	8	16	8	4	8	16	4	4	10.6 (6.5-17.0)
Infected	5	14	18-323	32	16	4	16	32	4	16	4	4	4	4	9.2 (4.8-17.6)
			18-334-X	16	16	4	32	8	4	32	8	4	16	16	10.6 (5.9-18.9)
Infected	12	61	18-323	256	128	64	32	64	32	64	32	64	32	64	59.7 (36.5-97.7)
			18-334-X	32	64	64	16	32	64	8	32	32	32	32	32.0 (20-51.7)

^a In each group of mice the titres towards the two *B. pertussis* strains are tabulated to allow vertical comparisons of agglutination levels towards 18-323 and 18-334 in individual sera.

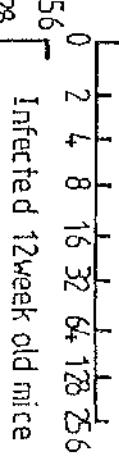
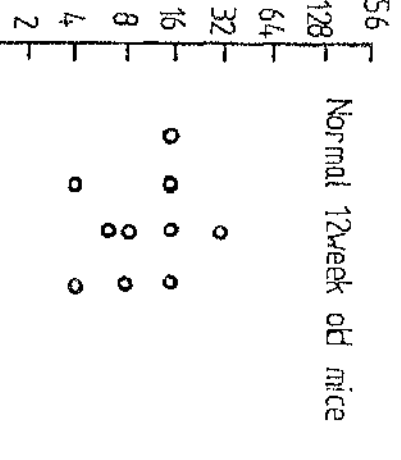
Figure 9. Correlation of reciprocal agglutination titres for
18-323 and 18-334 in the individual groups of table 11.
(X-mode of both strains were used.)

Reciprocal agglutination titre of 18-334

a



b



Reciprocal agglutination titre of 18-323

From statistical tables the critical values of P at the 5% level are 0.66 for Fig 6a and 0.632 for the others. Overall there was no significant correlation in reciprocal agglutination titres for the two strains of B. pertussis.

2.2 Immune response to LPS

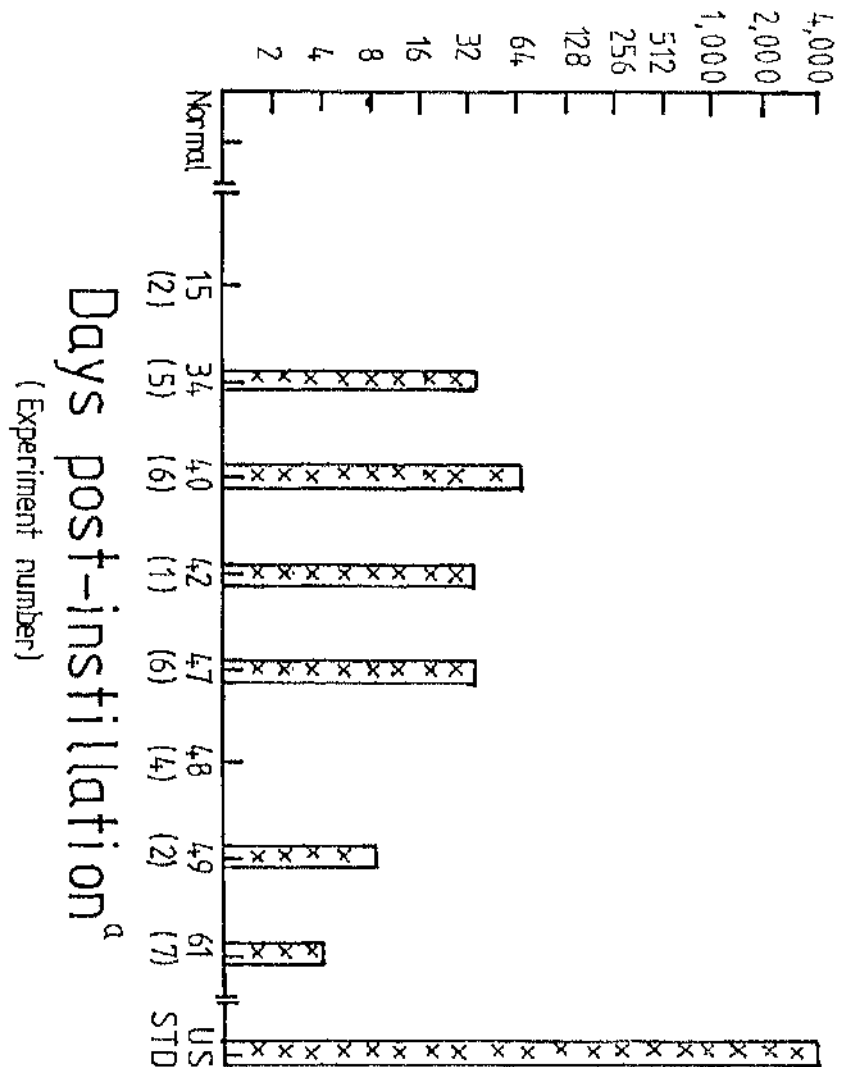
Since LPS is a prominent surface antigen (Aprile and Wardlaw, 1973) of B. pertussis, a possible immune response to this component in the pooled serum and TBW samples was investigated. This was done by determining the ability of these fluids to agglutinate horse RBC which had been coated with B. pertussis LPS (Figure 10).

The U.S. Standard Antipertussis serum which was included as a reference had a reciprocal titre of 4000, a lower titre than found in the agglutination test (16,000) with whole bacteria. Pooled convalescent sera had no demonstrable ($<1/1$) anti LPS at any of the sampling times despite having a bacterial reciprocal agglutination titre of 40-60 and having 50-100 times more protein than TBW. Pooled normal and acute phase sera had no detectable anti LPS.

Pooled day 34 convalescent TBW had a reciprocal anti LPS titre of between 32-64 which persisted to around day 47 post-instillation. By day 61 post-instillation the reciprocal titre in pooled TBW had dropped to 4. For unexplained reasons pooled day 48 convalescent TBW had no demonstrable antibody to LPS. However, this could indicate an inconsistent response to LPS during the sublethal pulmonary infection. Pooled normal and day 15 acute phase TBW lacked demonstrable antibody to LPS.

Figure 10. Reciprocal anti-LPS titres of pooled TBW from normal and B. pertussis infected mice obtained at different times post-instillation. The U.S. Standard Antipertussis Serum was used as a reference.

Reciprocal anti-LPS titre



^a Note this is not a linear scale

2.3 Immune response to F-HA

F-HA is another prominent surface component of B. pertussis (Sato, 1979). Therefore it might be expected to induce an immune response during a pulmonary infection. In this investigation, the anti-F-HA titre was determined in the pooled serum and TBW from normal and B. pertussis infected mice, with the U.S. Standard Antipertussis serum used as a reference.

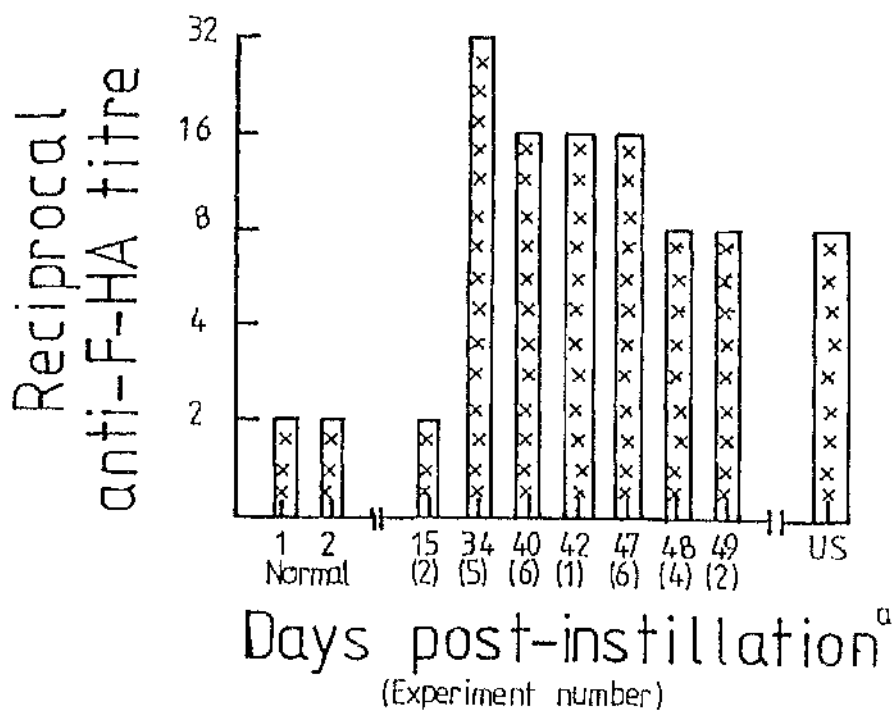
The latter, as shown in Figure 11, had a reciprocal anti-F-HA titre of 8. Pooled day 34 convalescent serum had a reciprocal anti F-HA titre of 32 which by day 49 had dropped to 8. Thus whereas in the bacterial agglutination tests, the reciprocal titre of the U.S. Standard Antipertussis serum was 300 fold greater than pooled convalescent serum, it was 4 fold lower than pooled day 34 convalescent serum and equal to pooled day 49 convalescent serum. Pooled convalescent TBW had no detectable anti-F-HA but it must be remembered that this fluid is highly diluted in its protein content compared with serum.

Pooled normal sera had a reciprocal anti-F-HA titre of 2 as did the pooled day 15 acute phase serum. Again, pooled normal sera appeared to have a natural antibody which reacted with a surface component of B. pertussis and did not exhibit a booster response after infection, since pooled day 15 acute phase serum showed no rise. Pooled normal and acute phase TBW had no detectable anti-F-HA.

2.4 Immune response to HLT

Anti-HLT antibodies do not develop in human whooping cough

Figure 11. Reciprocal anti-F-HA titres of pooled sera from normal and B. pertussis infected mice obtained at different times post-instillation. The U.S. Standard Antipertussis Serum was used as a reference.



^a Note this is not a linear scale

and Maitland, (Evans 1939) and Standfast (1951) was unable to establish a virulence role for HLT in the murine lethal pulmonary infection. However, the possible development of anti-HLT after a sublethal pulmonary infection in mice does not appear to have been investigated.

As shown in Table 12 the U.S. Standard Antipertussis serum, even undiluted, did not show the slightest sign of neutralising the test dose of HLT for 6 out of 6 suckling mice developed haemorrhagic oedema, the same as in the toxin-alone group. However there was available in the department a known active anti-HLT containing rabbit hyperimmune serum which had been prepared by intensive immunization as described in Materials and Methods. This serum was designated I.L. and had an anti-HLT titre of 120 units ml^{-1} . This antitoxin was able to neutralise the test dose of HLT with none of the six suckling mice developing haemorrhagic oedema.

Pooled convalescent serum showed no detectable anti-HLT action and neither did pooled convalescent TBW (Table 12). Pooled normal and acute phase serum also had no detectable anti-HLT and similarly neither did pooled normal and acute phase TBW. Thus if any anti-HLT had been produced it was below the level of detection by the system used.

2.5 Crossed-over immunoprecipitation

Immunoprecipitation is one of the procedures recommended for serological diagnosis of pertussis in children (Aftandeliens and Connor, 1973). This technique also offers the possibility of detecting immune responses to additional antigens in the bacteria

Table 12 : Anti-HLT in pooled sera and TBW from normal and *B. pertussis* infected mice obtained at different times post-instillation. The U.S. Standard Antipertussis Serum and rabbit hyperimmune antiserum with 120 antitoxin units ml⁻¹ were used as references

Type of Mouse and (Date of Sampling)	Expt. No.	Anti-HLT activity in ^b		Toxin alone controls
		TBW	Sera	
N ^a	1	4/4	3/3	6/6
N	2	4/4	3/3	2/2
I ^a (15)	2	3/3	3/4	6/6
I (34)	5	2/3	3/3	6/6
I (40)	6	2/2	3/3	6/6
I (42)	1	3/3	3/4	6/6
I (47)	6	3/4	3/4	6/6
I (48)	4	3/3	3/4	6/6
I (49)	2	4/4	4/4	6/6
I (61)	7	N.T. ^a	2/2	2/2
U.S. Standard Antipertussis Serum			6/6	6/6
Rabbit hyperimmune antiserum with 120 antitoxin units ml ⁻¹			0/6	6/6

a N = Normal; I = Infected; N.T. = Not tested

b Results are expressed as No. of suckling mice with haemorrhagic area/
total No. challenged.

which might not be exposed on the cell surface. This particular method of immunoprecipitation was chosen because of its sensitivity. Two different sonicated B. pertussis antigen preparations were used; 18-323 X mode and 18-334 C mode, to see if the antibodies responsible for the precipitation were directed against X mode specific virulence factors.

As shown in Plate 6, the U.S. Standard Antipertussis serum gave at least 2 strong lines against both X and C mode organisms. Pooled convalescent serum sampled from day 40 onwards gave single lines against X and C mode organisms. Thus the immune response detected by this method was not directed against exclusively X mode specific virulence factors.

Pooled day 34 convalescent serum gave no precipitin lines, against either antigen. No pooled convalescent TBW gave any precipitin lines against the antigen preparation. Pooled normal and day 15 acute phase serum also gave a negative result against both antigens as did pooled normal and day 15 acute phase TBW.

2.6 Quantitative Immunoelectrophoresis

This method also was used to investigate the immune response in pooled samples of serum and TBW to antigens in sonicated B. pertussis. It has the advantage of being more sensitive than crossed-over immunoelectrophoresis and gives a "profile" of the immune response rather than a single precipitin line.

The crossed immunoelectrophoretic pattern of sonicated B. pertussis reference antigen run against corresponding "Hertz"

Plate 6 : Crossed-over immunoprecipitation of pooled sera and TBW from normal and B. pertussis infected mice obtained at different times post-instillation. The U.S. Standard Antipertussis Serum was used as a reference. Sonicated B. pertussis strains 18-323 and 18-334 were employed as the antigens

	N ^a	N	D15 ^a	D15	D34	D34	D40	D40	D42	D42	US ^b
	TBW	SERA	TBW	SERA	TBW	SERA	TBW	SERA	TBW	SERA	STD
Row 1											18-323 X-Mode
2											18-334 C-Mode
	D47	D47	D48	D48	D49	D49	D61	D61	D61	D61	US
	TBW	SERA	TBW	SERA	TBW	SERA	TBW	SERA	TBW	SERA	STD
3											18-323 X-Mode
4											18-334 C-Mode

a : N = Normal; D = Day

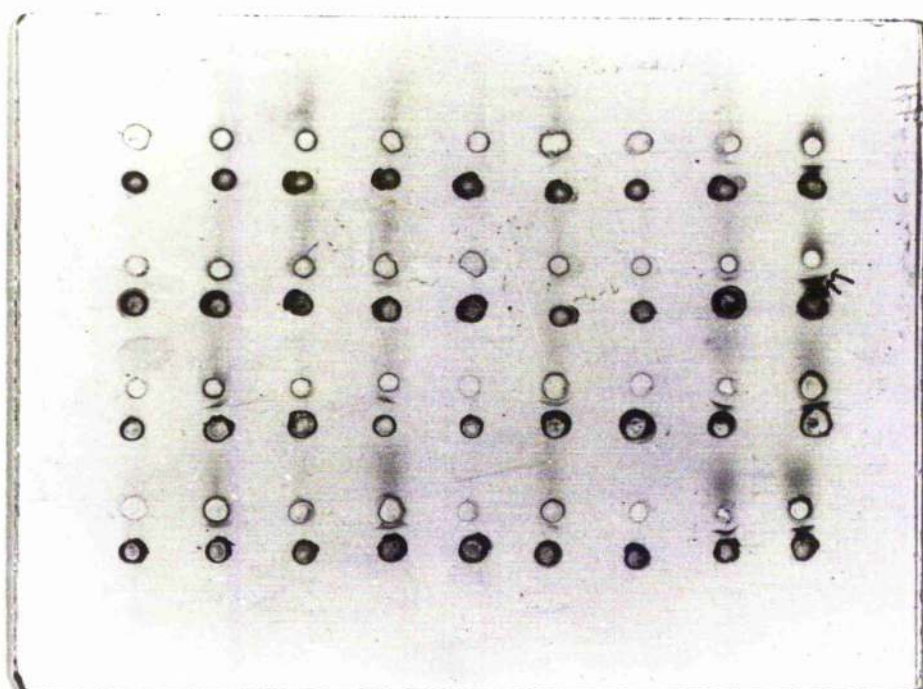
b : U.S.STD = U.S. Standard Antipertussis Serum

Rows 1 and 2: Have the same pooled serum or TBW in the top wells

Rows 3 and 4: Have the same pooled serum or TBW in the top wells
which are different from rows 1 and 2

Rows 1 and 3: Have 18-323 X-mode sonicate in the bottom wells

Rows 2 and 4: Have 18-334 C-mode sonicate in the bottom wells.

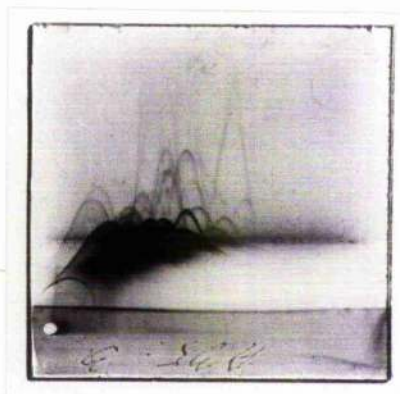


Arrows indicate the two Lines formed between
 U.S. Standard Antipertussis Serum and sonicated 18-334 C-mode B. pertussis

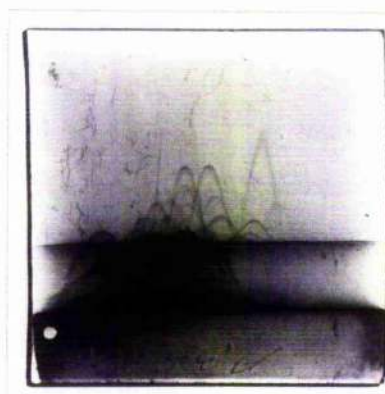
Plate 7 : Crossed immunoelectrophoresis of sonicated B. pertussis reference antigen run against corresponding rabbit antiserum in the second dimension gel with the intermediate gel containing diluent, U.S. Standard Antipertussis serum, or pooled normal, acute or pooled day 49 convalescent serum.

- a = NaCl in intermediate gel
- b = U.S. Standard Antipertussis serum
- c = pooled day 49 convalescent sera
- d = pooled acute phase sera
- e = pooled normal sera

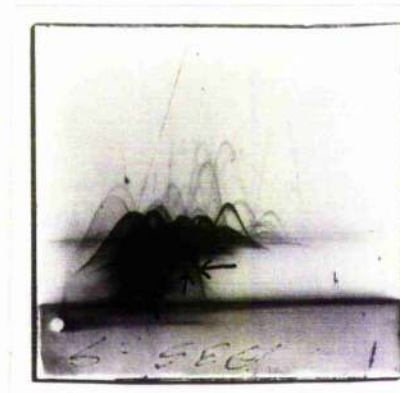
a.



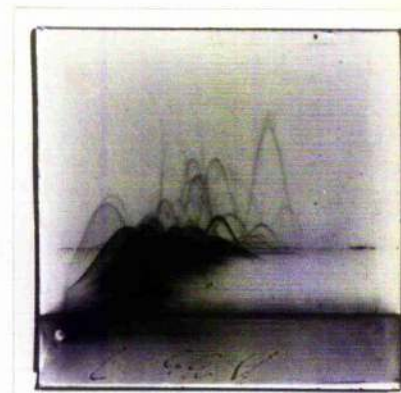
b.



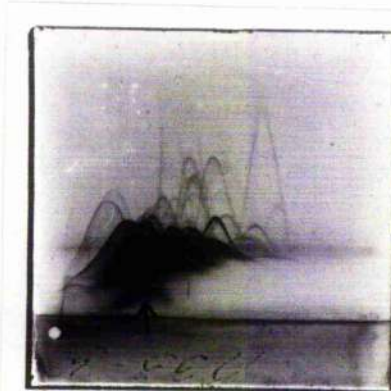
c.



d.



e.

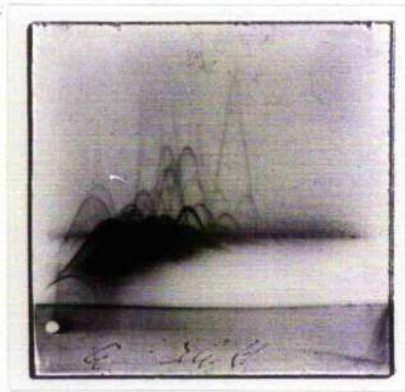


Arrows indicate peaks formed by pooled day 49 convalescent serum, pooled acute or pooled normal serum in the intermediate gel.

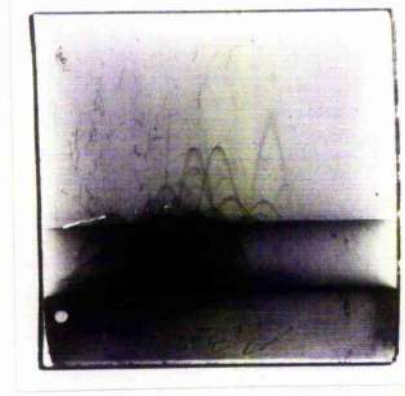
Plate 8 : Crossed immunoelectrophoresis of sonicated B. pertussis reference antigen run against corresponding rabbit antiserum in the second dimension gel with the intermediate gel containing diluent, U.S. Standard Antipertussis serum, or pooled normal, acute or pooled day 49 convalescent TBW.

- a = NaCl in intermediate gel
- b = U.S. Standard Antipertussis serum
- c = pooled day 49 convalescent TBW
- d = pooled acute phase TBW
- e = pooled normal TBW

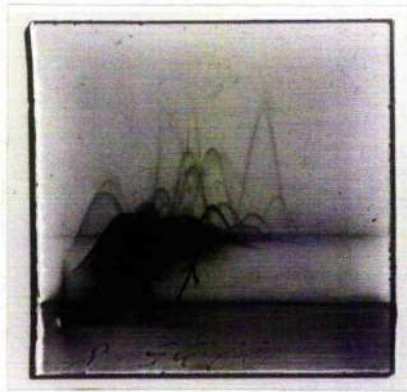
a.



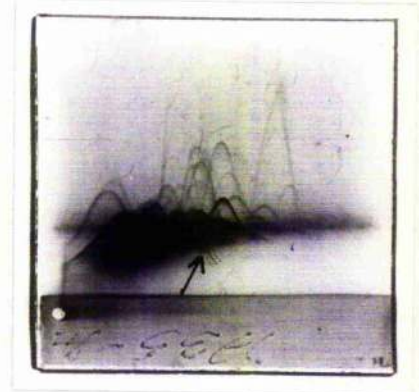
b.



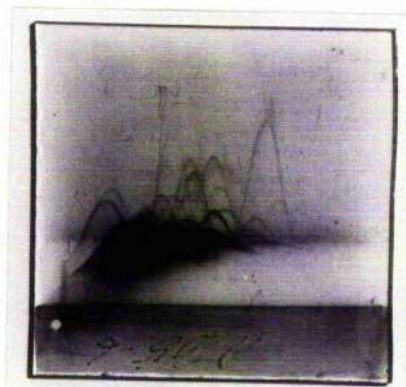
c.



d.



e.



Arrows indicate peaks formed by pooled day 49 convalescent TBW or pooled acute phase TBW in the intermediate gel.

rabbit antiserum (see Materials and Methods) in the second dimension gel was obtained when NaCl was placed in the intermediate gel (Plate 7a). A complex pattern of peaks was found in the intermediate gel when the U.S. Standard Antipertussis was incorporated in it (Plate 7b).

Four precipitin lines were formed when pooled day 49 convalescent sera was incorporated in the intermediate gel (Plate 7c) and two precipitin lines were formed in the intermediate gel with pooled acute phase sera incorporated (Plate 7d). Pooled normal sera formed 1 precipitin line (Plate 7e). No precipitin line was formed in the crossed immunoelectrophoresis of pooled normal and acute phase TBW.

The crossed immunoelectrophoretic pattern of sonicated B. pertussis reference antigen run against rabbit antiserum in the second dimension gel with NaCl and U.S. Standard Antipertussis serum in the intermediate gel are presented again in Plates 8a and 8b respectively. When pooled day 49 convalescent TBW was incorporated into the intermediate gel, three precipitin lines were formed (Plate 8c). Two precipitin lines were formed by pooled acute phase TBW (Plate 8d) were undetected in crossed over immunoelectrophoresis. Pooled normal TBW did not precipitate any lines when incorporated into the intermediate gel (Plate 8e).

As well as quantitating the number of immune responses present to sonicated B. pertussis reference antigen, this immunoelectrophoresis was more sensitive than crossed over immunoelectrophoresis which was unable to detect antibodies in pooled normal and acute phase sera. It was also able to detect an immune response in pooled acute and convalescent phase TBW where neither agglutination, haemagglutination inhibition test or crossed-over immunoprecipitation did.

2.7 Bactericidal activity

The possible bactericidal activity of mouse serum and TBW was investigated primarily because it might adversely affect the results of passive protective tests done by the "intranasal mix" method (North, 1946). . Involved in this method is the preparation of mixtures of pooled serum and TBW with live B. pertussis which are then held for up to 1 h at room temperature in the course of administering the challenge. During this time, the bacteria might die. To explore this question and also to increase the chance of demonstrating bactericidal activity, mixtures of serum or TBW with B. pertussis strain 18-323 were incubated for 90 min at 37°C and viable counts made.

The results of Table 13 show that the mean dose inoculated into each sample was 33 cfu per sample volume (0.05 ml) and that after 90 min incubation at 37°C all the mixtures, in fact, yielded an increase in viable count. This indicated that the bacteria far from being killed in the mixtures were able to multiply. It was therefore concluded that no reduction in viability of challenge would occur over the time scale and under the conditions in which mixtures of sera and TBW with B. pertussis were held while performing the intranasal challenge.

2.8 Passive protective activity towards sublethal intranasal infections of B. pertussis

All of the above described immunological assays on the pooled convalescent sera and TBW had been done in vitro and therefore did not

Table 13 : Bactericidal activity of pooled sera and TBW from normal and B. pertussis infected mice at different times post-institution

Test Fluid	Viable count per 0.05 ml after 90 min incubation at 37°C in fluids from mice that were:-			
	Normal	day 15	day 42	day 49
Serum	77	58	75	81
TBW	58	39	58	49
Control C.A.A. at 90 min	53			
Mean dose inoculated at time zero	33			

provide direct information on protective activity against infection. It therefore seemed appropriate at this stage in the investigation to study serum and TBW for protective activity against SLIN challenge. For this purpose the protection was assessed by both lung pathology and culture score at day 14 (infection peak) post-instillation of challenge.

A quantitative comparison between SLIN passive protective activity of pooled sera and TBW was made expressing both in terms of U.S. Standard Antipertussis serum. The passive protective activity of the pooled samples was also assessed by the IC infection route. This allowed the comparison between the passive protective potency of pooled samples against the SLIN and IC infections.

An initial experiment was carried out by the SLIN/mix method to determine if in fact pooled convalescent sera and TBW were passively protective and, if so, if heating for 30 min at 36°C to remove complement reduced this passive protective activity.

Table 14a records the individual lung pathology and culture score for each mouse and also the significance by the Mann-Whitney U-test of each group result compared to that of the relevant lung pathology or culture score of the challenge alone group. Table 14b records the proportion of the responses ≥ 2 and also the significance of each group compared to the challenge alone lung pathology or culture by the 2 x 2 contingency tables by Finney et al (1963). Appendix 7 gives U_1 and U_2 values for Table 14a. Pooled day 49 convalescent serum significantly reduced lung pathology and culture score by the Mann-Whitney test and highly significantly by the 2 x 2 contingency tables of Finney et al (1963), when compared to challenge

Table 14a : Sublethal intranasal passive protective activity of pooled sera and TBW from normal and infected mice, and the effect of heating the samples for 30 min at 56°C before use

Type of Mouse ^a and (Day of sampling)	Pooled sample	Individual mouse score			
		Unheated fluid		Heated 30 min at 56°C	
		Pathology	Culture	Pathology	Culture
N	TBW	1112 (NS) ^b 2333	0022 (NS) 2344	N.T.	N.T.
I (49)	TBW	0000 (*) 0112	0000 (*) 0002	0001 (*) 1122	0001 (*) 1122
N	Sera	1122 (NS) 2344	0144 (NS) 44	3333 (NS) 4444	3444 (NS) 44
I (49)	Sera	0000 (*) 0002	0000 (*) 0004	0000 (*) 0012	0000 (*) 3
Challenge alone		0233 3334	3344 44		

a N = Normal; I = Infected

b Symbols in parenthesis express the significance of the difference by the indicated scoring method and the corresponding scoring method in the challenge alone group assessed by the Mann-Whitney U-test.
NS = Not significant; * = significant at the 5% level.

Table 14b : SLIN mix data as shown in table 14a but tabulated as the proportion of animals with scores of 2 or more before or after heating the samples for 30 min at 56°C

Type of mouse ^a and (Day of sampling)	Pooled sample	Passive protective activity (No of mice with ≥ 2 score/total)			
		Unheated fluid		Heated 30 min at 56°C	
		Pathology	Culture	Pathology	Culture
N	TBW	5/8 (NS) ^b	6/8 (NS)	N.T.	N.T.
I (49)	TBW	1/8 (**)	1/5 (**)	2/8 (*)	2/8 (*)
N	Sera	8/8 (NS)	7/8 (NS)	6/8 (NS)	6/6 (NS)
I (49)	Sera	1/8 (**)	1/8 (**)	1/8 (**)	1/5 (**)
Challenge alone		7/8	6/6		

a N = normal; I = infected

b Symbols in parenthesis express the significance of the difference between the indicated scoring method and the corresponding scoring method in the challenge alone group as assessed by the 2x2 contingency tables of Finney et al (1963).

NS = Not significant and * = significant at the P = 5% level. ** = highly significant at the P = 1% level.

controls. Heating for 30 min at 56°C had no effect on this passive protective activity since heated convalescent pooled day 49 serum still give the same degree of significant reduction of lung pathology and culture by both analyses.

Pooled day 49 convalescent TBW also significantly reduced lung pathology and culture score by the Mann-Whitney test and highly significantly by the 2 x 2 contingency tables when compared to challenge alone group. Heating for 30 min at 56°C again had no effect on passive protective activity as measured either by lung pathology or culture score of mice when compared to challenge alone.

Pooled normal serum did not reduce lung pathology and culture from that found in the challenge alone by either the Mann-Whitney test or the 2 x 2 contingency tables. This was also true for pooled normal TBW.

This passive protective activity of pooled day 49 convalescent sera and TBW appears to be independent of complement since heating for 30 min at 56°C had no effect.

Referring to the two alternative methods of statistical analysis, it may be stated that, in general they yielded similar conclusions, ie where the difference was significant by the Mann-Whitney U test it was also significant by the 2 x 2 contingency tables. The same was true where the result was insignificant. However in a few instances where the group size was small, the Mann-Whitney U-test seemed less sensitive than the 2 x 2 contingency tables in detecting a significant difference. Table 15 gives such an example.

In light of the above, the results of subsequent SLIN passive protection tests were presented as proportions of ≥ 2 score and

Table 15 : Example of reduced sensitivity of Mann-Whitney U-test compared to the 2 x 2 contingency tables when determining sublethal intranasal passive protective activity with a small number of observations

Type of mouse ^a and (Day of sampling)	Pooled sample	Individual mouse score Pathology	Type of mouse ^a and (Day of sampling)	Pooled sample	Passive protective activity No. with 2 pathology No. infected
N	TBW	02344 (NS) ^b	N	TBW	4/5 (NS) ^b
I (49)	TBW	01113 (NS)	I (49)	TBW	1/5 *
Challenge alone		02244	Challenge alone		4/5

a N = Normal; I = Infected

b Symbols in parenthesis express the significance of the difference between the indicated pathology score and the challenge alone pathology score as assessed by the Mann-Whitney U-test.
NS = Not significant and * = significant at the P = 5% level.

a N = Normal; I = Infected

b Symbols in parenthesis express the significance of the difference between passive protective activity of indicated proportion and the challenge alone group as assessed by the 2 x 2 contingency tables of Finney et al (1963). NS = Not significant and * = significant at the P = 5% level.

analysed by the 2×2 contingency tables. For completeness of record however, the corresponding individual mouse scores are tabulated in the Appendix and analysed by the Mann-Whitney U-test.

2.8.1 Sublethal intranasal passive protective activity development in pooled sera and TBW

To identify quantitative trends in the development of SLIN passive protective activity of the pooled sera and TBW obtained in Expt 1-8, SLIN passive protective activity was titrated using both reduction in significant lung pathology and culture score as criteria. Appendix 8a-d gives the results of the individual titration of the pooled samples for both lung pathology and culture score. From each titration, the $PD_{50} \text{ ml}^{-1}$ and 95% C.L. were obtained using the U.S. Standard Antipertussis serum as the reference serum of relative potency 1.0 (Figure 12).

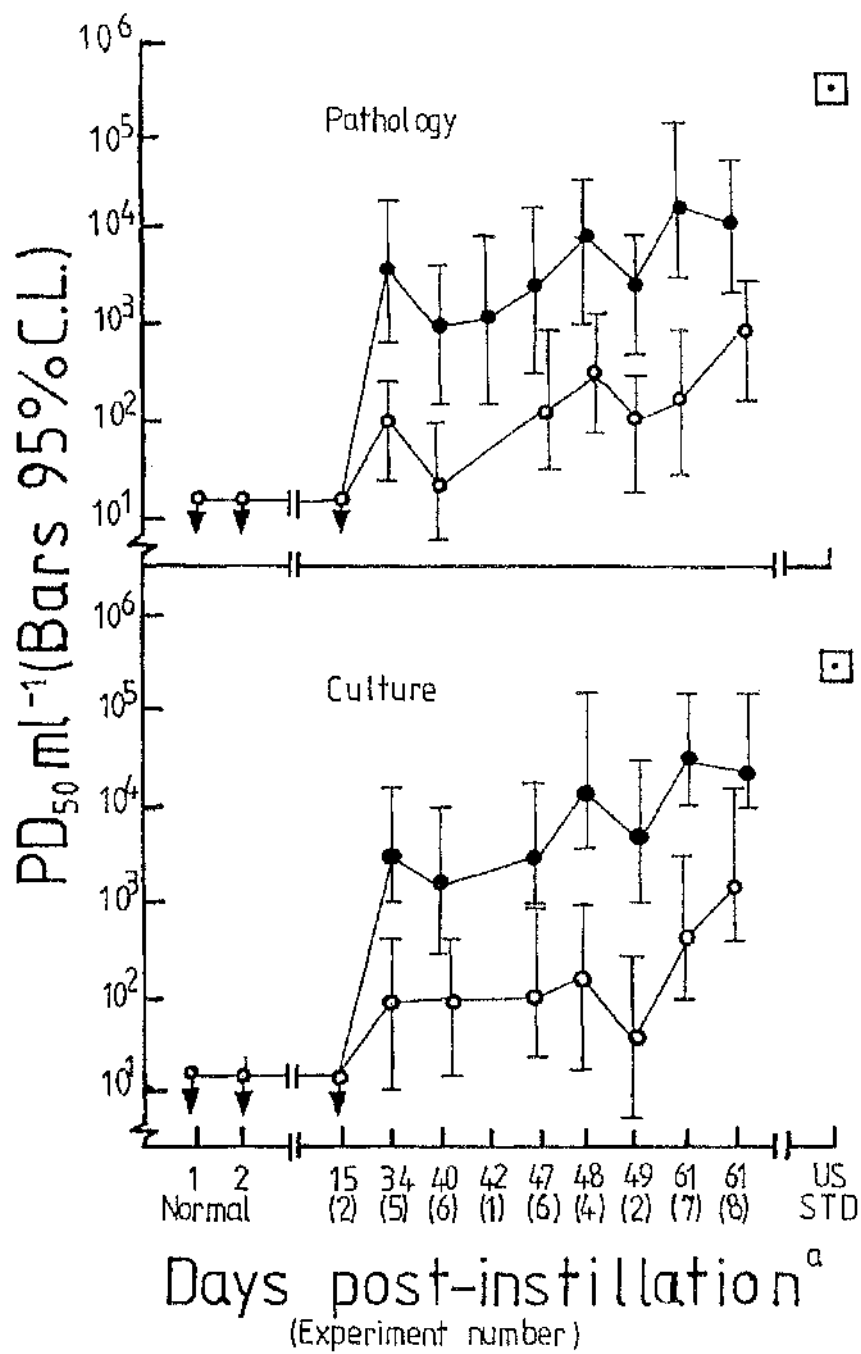
The U.S. Standard Antipertussis serum had an SLIN passive protective activity by the lung pathology criterion of 570,000 $PD_{50} \text{ ml}^{-1}$; by lung culture the value was 440,000. Pooled day 34 convalescent sera had developed SLIN passive protective activity of 6,666 $PD_{50} \text{ ml}^{-1}$ by lung pathology and 5,000 $PD_{50} \text{ ml}^{-1}$ by lung culture. By day 61 activity had increased to between 13,500-33,000 $PD_{50} \text{ ml}^{-1}$ as determined by lung pathology and 40,000-50,000 $PD_{50} \text{ ml}^{-1}$ as determined by lung culture.

Pooled day 34 convalescent TBW had also developed SLIN passive protective activity and prevented development of lung pathology ($PD_{50} \text{ ml}^{-1} = 125$) and lung culture ($PD_{50} \text{ ml}^{-1} = 100$). This again increased by day 61 where pooled convalescent TBW had between 222-1000

Figure 12 : Development of sublethal intranasal passive protective activity in serum and TBW of B. pertussis intranasally infected mice. Note that the abscissa, as on other Figures, is not a linear scale and the diagram summarises data from several independent experiments. The U.S. Standard Antipertussis Serum was used as the reference preparation for calculating 95% C.L.

- = U.S. Standard Antipertussis serum
- = pooled convalescent sera
- = pooled convalescent TBW
- ⊙
↓ = pooled normal and acute phase serum and TBW

The individual mouse data on which this figure is based are recorded in Appendices 8a-d.



^a This is not a linear scale

$PD_{50} \text{ ml}^{-1}$ as determined by lung pathology and 666-2,000 $PD_{50} \text{ ml}^{-1}$ as determined by lung culture.

Pooled normal serum had no detectable passive protective activity in preventing lung pathology and lung culture, ie $< 20 PD_{50} \text{ ml}^{-1}$, the limit of detection. By day 15 post-instillation there was still no detectable SLIN passive protective activity. A similar absence of detectable passive protective activity was found in pooled normal and day 15 acute phase TBW.

Thus mice which were convalescent from a sublethal infection had developed SLIN passive protective activity at least by day 34 of infection in both the sera and TBW. This development continued until at least day 61 post-instillation. Pooled convalescent sera were approx 40 times more potent than pooled convalescent TBW and the U.S. Standard Antipertussis serum was, in turn, 20 times more potent than pooled day 61 convalescent serum both in reducing lung pathology and culture.

2.9 Passive protective activity towards intracerebral infections of *B. pertussis*

Since convalescent serum and TBW had easily detected passive protective activity by the SLIN/mix test, it was obviously of interest to see if the same specimens would also protect mice against IC challenge. Moreover, it may be noted that the number of cfu in the challenge dose of strain 18-323 is quite similar in both infection routes. However 10^5 cfu by the IN route is approximately 1 LD_{25} whereas 10^5 cfu by the IC route is $> 100 LD_{50}$. So the IC/mix

passive protection test is a more severe trial of antibody protective activity than the SLIN/mix passive protection test.

In these IC passive protection tests the samples were given as a 1 in 2 dilution with challenge organisms which were the IC virulent 18-323 strain supplied by Dr. F. Sheffield except when pooled samples were tested from expt 1 (Table 17) where the 18-323 strain supplied by Dr. Pittman was used. From this experiment it was obvious that the IC virulence of this strain was falling since in the challenge alone group given a dose which should have been 100 LD₅₀ finished with only 7 out of 10 dead.

Intracerebral passive protective activity of the U.S. Standard Antipertussis serum was titrated and found to contain 1650 PD₅₀ ml⁻¹ against >100 LD₅₀ (Table 16). This was a 300-fold smaller value than against 1 LD₂₅ in the SLIN infection. Turning to the results with pooled convalescent sera and TBW (Table 17), it will be seen that no IC-passive-protective activity was detected in any of the experimental samples. Even when only 1 LD₇₀ was given with pooled day 42 convalescent sera and TBW, 7 out of 10 and 8 out of 10 mice died respectively. Therefore no passive protective activity was detected for pooled day 42 convalescent samples against a challenge equivalent to 3 LD₂₅ of the SLIN challenge. However these were the only samples receiving 1 LD₇₀. The other pooled convalescent samples received >100 LD₅₀ challenge.

Pooled normal and acute phase samples also had no detectable passive protective activity against IC challenge of 1 LD₇₀ or >100 LD₅₀.

Table 16 : Intracerebral passive protective activity of U.S. Standard
Antipertussis Serum

Passive protective activity Dilutions					$PD_{50} ml^{-1}$	Challenge Dose
($\frac{\text{Deaths}}{\text{Total Challenged}}$)						(LD_{50})
Neat	1/20	1/200	1/2000	1/ α		
0/10	5/10	9/10	10/10	10/10	1650	> 100 LD_{50}

Table 17 : Intracerebral passive protective activity of pooled sera and TBW from normal and B. pertussis infected mice obtained at different times post-instillation

Type of mouse ^a and (Day of sampling)	Expt No.	Passive protective activity (Deaths/Total challenged)		Challenge alone ^b
		TBW (Undiluted)	Serum (Undiluted)	
N	1	8/10	6/10	7/10
N	2	8/8	8/8	8/8
I (15)	2	7/8	8/8	8/8
I (34)	5	8/8	8/8	7/8
I (40)	6	8/8	7/8	7/8
I (42)	1	8/10	7/10	7/10
I (47)	6	7/8	6/8	7/8
I (48)	4	7/8	7/8	7/8
I (49)	2	7/8	8/8	8/8
I (61)	7	10/10	10/10	10/10
I (61)	8	10/10	10/10	10/10

a N = Normal; I = Infected.

b The LD₅₀ of challenge used to test pooled sera and TBW collected in Expt 1 was 2 LD₅₀ while in all the other tests it was greater than 100 LD₅₀. Nevertheless in the challenge doses the c.f.u. was always around 1×10^5 c.f.u.

2.10 Absorption of pooled sera with Phase I and Phase IV B. pertussis

Since the SLIN passive protective activity developed in the sera of mice convalescent from a pulmonary infection, it was of interest to see if this activity was directed against surface virulence component(s) specific to Phase I B. pertussis or towards surface components found also on Phase IV avirulent organisms.

The criterion used to determine the minimal dose of B. pertussis with which to absorb pooled convalescent sera was that dose of Phase I 18-323 which absorbed all agglutinins. This was determined by absorbing 1 ml aliquots of a pooled convalescent serum with a range of doses from 10 o.u. to 200 o.u. Phase I B. pertussis and then titrating the agglutination titre in the absorbed samples (Table 18).

Absorption with 200 o.u. Phase I B. pertussis per ml of serum removed all agglutinins to both agglutinating strains (18-323 and 18-334). With smaller absorption doses, the residual reciprocal agglutination titre showed less decline with both agglutinating strains. The pooled serum absorbed with 10 o.u. Phase I B. pertussis ml⁻¹ had a reciprocal agglutination titre of 16 for both 18-323 and 18-334 agglutinating strains which was only a factor of 2 less than unabsorbed pooled convalescent sera which had a reciprocal agglutination titre of 32 towards both agglutinating strains.

Plate 9 shows crossed-over immunoelectrophoresis of the absorbed sera using the antigenic preparations of B. pertussis Phase I 18-323 (Row 1 and 2) and Phase IV D30042 (Row 3 and 4). Precipitin lines were absent between pooled convalescent sera absorbed with 200 o.u. to both antigenic preparations of B. pertussis (well A of

Table 18 : Reciprocal agglutination titre of pooled day 48
convalescent sera either unabsorbed or absorbed with
Phase I B. pertussis at various doses. Agglutinins were
tested with B. pertussis strains 18-323 and 18-334

Absorption treatment (ml ⁻¹) of sera (with <u>B. pertussis</u> 18-323) (o.u.)	Reciprocal agglutinating titre ^a with strain	
	18-323	18-334
Nil (unabsorbed)	32	32
10	16	16
20	16	16
50	4	8
100	4	4
200	< 1	< 1

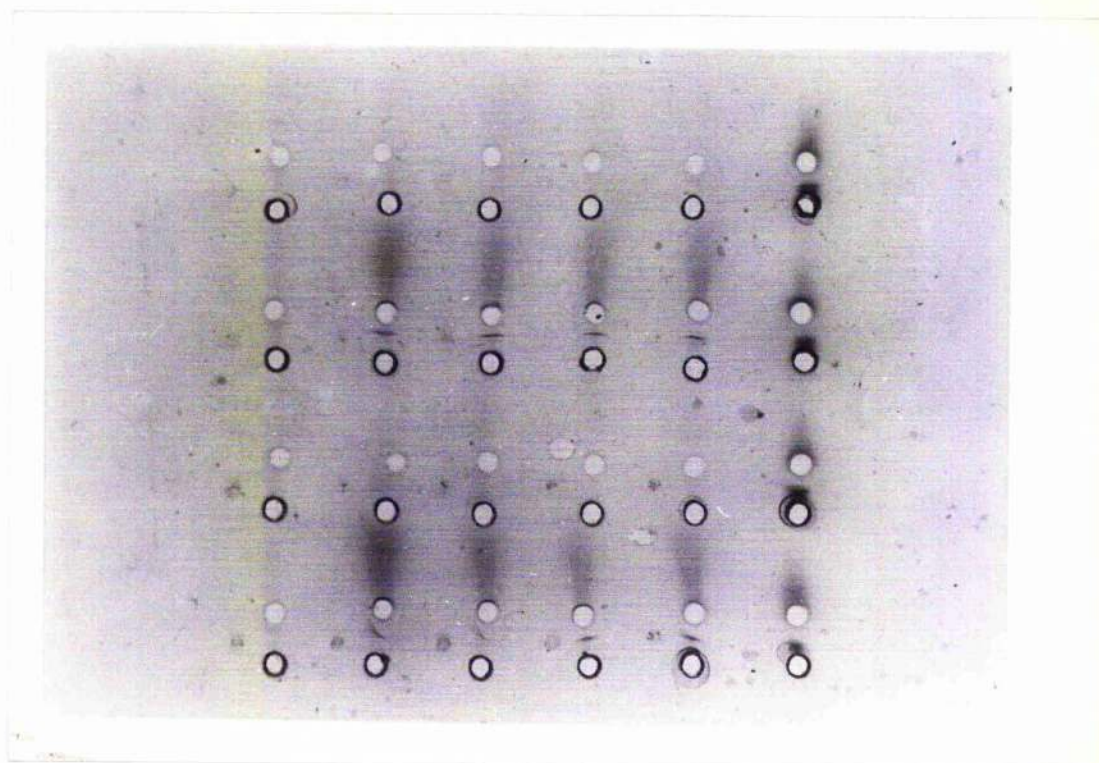
a All titrations were done in duplicate on the same day.

Consistent results for each value were obtained.

Plate 9 : Crossed-over immunoelectrophoresis of pooled sera from mice at day 48 of a pulmonary infection of B. pertussis strain 18-323 and the same absorbed with either various doses of Phase I or two different doses of Phase IV B. pertussis. The antigen preparations used were B. pertussis Phase I 18-323 and Phase IV D30042

	A	B	C	D	E	F
1						Antigen preparation Phase I sonicate
2						
3						Antigen preparation Phase IV sonicate
4						
Antisera in rows 2 and 4	Antisera in rows 1 and 3					
A = 200 o.u. Absorbed sera	A 200 o.u. Phase I absorbed sera					
B = 100 o.u. " "	B 1000 o.u. " " "					
C = 50 o.u. " "	C 200 o.u. Phase IV absorbed sera					
D = 20 o.u. " "	D 1000 o.u. " " "					
E = 10 o.u. " "	E Normal sera					
F = U.S. Standard Antipertussis Serum.	F U.S. Standard Antipertussis Serum.					

a Antigenic preparations were made by scraping the growth of a 24 h puddle plate to make a thick suspension in C.A.A. then sonicating for 6 x 20 sec.



rows 2 and 4). However precipitin lines were found between pooled convalescent serum absorbed with 100 o.u. B. pertussis Phase I 18-323 and both antigenic preparations of B. pertussis. The minimum dose of B. pertussis Phase I 18-323 or Phase IV D30042 used to absorb each ml of pooled convalescent serum was therefore 200 o.u. To ensure the removal of phase specific antibodies, aliquots of serum were absorbed (per ml) with 1000 o.u. of either B. pertussis Phase I 18-323 or Phase IV D30042. (Wells A to D of rows 1 and 3 confirm these absorptions with both Phase I and IV B. pertussis completely removing precipitins.)

Sublethal intranasal passive protective activity of the unabsorbed and absorbed pooled convalescent sera (all at 1/10 diln) were determined in two separate experiments. Normal serum was absorbed with 200 o.u. Phase I B. pertussis to see if absorption augmented the virulence of challenge. The results of both experiments are given as the proportion of significant lung pathology and culture score (Table 19) and the individual lung pathology and culture scores analysed by the Mann-Whitney U-test are in Appendix 9.

Unabsorbed pooled convalescent serum produced at least a significant reduction in pathology and culture score. However this pooled convalescent serum absorbed with either 200 o.u. or 1000 o.u. Phase I B. pertussis was not passively protective and there was no difference between both pathology and culture results found in mice given infection alone and those treated with pooled convalescent serum absorbed with both doses of Phase I B. pertussis.

Pooled convalescent serum absorbed with both doses of Phase IV remained passively protective, since it produced at least a significant

Table 19 : Effect of absorption with Phase I and Phase IV^a B. pertussis on sublethal intranasal passive protective activity of mouse convalescent sera: proportion of animals showing ≥ 2 score in their pathology and culture results of Experiments 1 and 2

Absorption treatment of sera with <u>B. pertussis</u> (o.u.)	Expt 1				Expt 2			
	Passive protective activity (No. ≥ 2 score)		(No. infected or uncontaminated cultures)		(No. ≥ 2 score)		(No. infected or uncontaminated cultures)	
	Pathology	Culture	Pathology	Culture	Pathology	Culture	Pathology	Culture
Nil	2/10 ** ^b	1/10 **	1/8 **	0/8 **				
200 o.u. Phase I	9/10 (NS)	7/10 (NS)	8/8 (NS)	6/8 (NS)				
1000 o.u. Phase I	10/10 (NS)	10/10 (NS)	8/8 (NS)	8/8 (NS)				
200 o.u. Phase IV	1/10 **	1/10 *	3/8 *	1/8 *				
1000 o.u. Phase IV	2/10 **	2/10 *	2/8 *	1/8 *				
Controls								
Infection alone	10/10	9/10	7/8	6/8				
Normal sera absorbed with 200 o.u.	9/10 (NS)	10/10 (NS)	N.T.	N.T.				

a Absorption with both Phases of B. pertussis was done with 2 different doses 200 o.u. and 1000 o.u.

b The significant difference between the difference from the infection alone group by the 2 x 2 contingency tables of Finney et al (1963). NS = Not significant; * = significant at the 5% level; ** = significant at the 1% level.

reduction in pathology and culture score compared to those found in the infection alone group. Pooled normal serum absorbed with 200 o.u. Phase I B. pertussis did not augment the virulence of the infection and mice treated with pooled normal absorbed serum did not give significantly different pathology or culture score from the infection alone.

To confirm that absorption with Phase I B. pertussis did remove SLIN passive protective activity from pooled convalescent sera and Phase IV B. pertussis did not, the absorption procedure was repeated and SLIN passive protective activity determined on two separate experiments (Table 20). Individual pathology and culture score analysed by the Mann-Whitney U-test are given in Appendix 10.

These results confirm that absorption of pooled convalescent sera with either 200 o.u. or 1000 o.u. B. pertussis Phase I removes SLIN passive protective activity as determined by both pathology and culture score. Whereas B. pertussis Phase IV absorption with either 200 o.u. or 1000 o.u. of pooled convalescent sera had no effect on the SLIN passive protective activity in that Phase IV absorbed sera remained passively protective by the SLIN route. Thus SLIN passive protective activity seems to be directed against Phase I specific surface antigens since even the 1000 o.u. dose of Phase IV did not remove SLIN/mix passive protective activity from pooled day 48 convalescent serum.

2.11 Passive protective activity of pooled convalescent sera and TBW given intranasally before challenge

North (1946) demonstrated mouse convalescent serum to be

Table 20 : Effect of absorption with Phase I and Phase IV B. pertussis on sublethal intranasal passive protective activity of mouse convalescent sera: proportion of animals showing ≥ 2 score in their pathology and culture results of Experiment 1 and 2

Absorption treatment of sera with B. pertussis (o.u.)	Passive protective activity (No. ≥ 2 score) (No. infected or uncontaminated cultures)			
	Expt 1		Expt 2	
	Pathology	Culture	Pathology	Culture
Nil	0/10 ** ^b	1/10 **	1/10 **	2/10 *
200 o.u. Phase I	7/9 (N.S.)	6/9 (N.S.)	9/10 (N.S.)	8/9 (N.S.)
1000 o.u. Phase I	6/9 (N.S.)	5/8 (N.S.)	9/10 (N.S.)	5/7 (N.S.)
200 o.u. Phase IV	0/9 **	0/9 **	1/10 **	3/10 *
1000 o.u. Phase IV	1/7 **	0/7 **	1/10 **	1/3 *
Controls				
Infection alone	6/6	5/5	6/7	4/5
Normal sera absorbed with 200 o.u. Phase I	9/10 (N.S.)	10/10 (N.S.)	8/8 (N.S.)	6/8 (N.S.)

a Absorption with both phases of B. pertussis was done with 2 different doses 200 o.u. and 1000 o.u.

b The statistical significance between the difference from the infection alone group by the 2 x 2 contingency tables of Finney et al (1963). NS = Not significant; * = significant at the 5% level; ** significant at the 1% level.

passively protective when administered intranasally up to 48 hours before a SLIN challenge. In terms of modern immunology this could be interpreted as indicating a possible role of IgE in SLIN passive protective activity of mouse convalescent serum if one accepts that homocytotrophic IgE would be the only immunoglobulin to persist on pulmonary epithelium over a 48 hour period. It was therefore of interest to establish if the pooled convalescent sera and also TBW obtained in the present investigation had this ability to protect passively when given intranasally up to 48 hours before a SLIN challenge.

In the first experiment (Table 21) when pooled convalescent sera at 1/20 dilution was given intranasally 48, 24 and 6 h before challenge, it was found to be as passively protective against pathology development as both dilutions: 1/20 and 1/200 given intranasally as a mixture with challenge. The individual lung pathology and culture scores are in Appendix 11. There was a highly significant reduction in pathology of all groups given pooled convalescent serum compared to the pathology of challenge alone by the 2 x 2 contingency tables of Finney et al (1963). However since no culture results were obtained (because of mould contamination) the experiment was repeated.

The pathology results in the second experiment were similar to the first with convalescent serum given 48 h before challenge significantly reducing pathology score. Convalescent serum (1/20) mixed with challenge produced a highly significant reduction in both pathology and culture and at a 1/200 dilution mixed with challenge there was a significant reduction in both pathology and culture. However the culture scores of mice given convalescent serum 48 h

Table 21 : Sublethal intranasal passive protective activity of pooled day 48 convalescent sera from B. pertussis infected mice when given intranasally 48, 24 and 6 h before a sublethal intranasal challenge and as a challenge + serum mixture

Time of administration of sera in relation to challenge (dilution)	Passive protective activity (No. \geq 2 score/No. infected or uncontaminated cultures)					
	Expt 1 ^a		Expt 2		Expt 3	
	Pathology	Culture	Pathology	Culture	Pathology	Culture
48 hours before (1/20)	0/5 ** ^c	- ^b	1/5 *	3/4 (NS)	0/8 **	0/4 **
24 hours before (1/20)	0/5 **	-	N.T.	N.T.	N.T.	N.T.
6 hours before (1/20)	0/5 **	-	N.T.	N.T.	N.T.	N.T.
Mixture (1/20)	0/5 **	-	0/5 **	0/5 **	4/8 (NS)	3/6 (NS)
Mixture (1/200)	0/5 **	-	1/5 *	1/5 *	4/7 (NS)	4/4 (NS)
Controls						
Challenge alone	8/10	-	5/5	5/5	7/8	5/5
Ether 2 days before	N.T. ^d	N.T.	5/5 (NS)	5/5 (NS)	7/8 (NS)	6/8 (NS)
CAA 2 days before	N.T.	N.T.	4/5 (NS)	5/5 (NS)	7/8 (NS)	5/7 (NS)
Normal sera 2 days before	N.T.	N.T.	4/5 (NS)	3/4 (NS)	6/8 (NS)	4/5 (NS)

^a c.f.u. of challenge of Expt 1 = 7×10^4 ; Expt 2 = 1.1×10^5 ; Expt 3 = 1.0×10^5 ;

^b Expt 1 had no culture results due to complete contamination

^c The statistical significance when compared to infection alone group by the 2 x 2 contingency tables of Finney et al (1963). NS = Not significant; * = significant at the 5% level; ** = significant at the 1% level.

^d NT = Not tested.

before challenge disagreed with the pathology results and were not significantly reduced.

This implied that the antibody which persisted for 48 h prevented pathological change induced by B. pertussis but was not involved in clearance of the organisms, since they were still present after 14 days. However when the experiment was again repeated, pooled convalescent serum given 48 hours before challenge reduced in a highly significantly fashion both the lung pathology and culture scores. Mice treated with 1/20 and 1/200 dilution of pooled convalescent serum mixed with challenge did not significantly reduce either lung pathology or culture in experiment 3 for reasons unknown. In all experiments mice given anaesthetic alone two days before challenge were no less susceptible to infection as the challenge alone mice.

The same procedure was followed with pooled day 48 convalescent TBW which had been x10 concentrated (Table 22). The individual lung pathology and culture scores are in Appendix 12. In the first experiment, pooled convalescent TBW at a 1/2 dilution did not significantly reduce pathology score when given 48 h before challenge. However when given 24 or 6 h before challenge it did highly significantly prevent pathology development. Mice given a 1/2 or 1/20 dilution of pooled convalescent TBW mixed with challenge had a highly significant reduction in lung pathology. The experiment was repeated to obtain culture results.

Pooled day 48 convalescent TBW given 48 h before challenge in experiment 2 produced a highly significant reduction in pathology and a significant reduction in culture. However pooled convalescent TBW

Table 22 : Sublethal intranasal passive protective activity of $10 \times$ concentrated pooled day 48 convalescent TBW from *B. pertussis* infected mice when given intranasally 48, 24 and 6 h before sublethal intranasal challenge and as challenge + TBW mixture

Time of administration of TBW in relation to challenge (dilution)	Passive protective activity (No. \geq 2 score/No. infected or uncontaminated cultures)			
	Expt 1 ^a		Expt 2	
	Pathology	Culture	Pathology	Culture
48 hours before (1/2)	3/5 (NS) ^c	- ^b	1/5 **	1/3 *
24 hours before (1/2)	0/5 **	-	3/5 (NS)	2/4 (NS)
6 hours before (1/2)	0/5 **	-	N.T.	N.T.
Mixture (1/2)	0/5 **	-	2/5 (NS)	2/3 (NS)
Mixture (1/20)	0/5 **	-	4/5 (NS)	3/4 (NS)
Controls				
Challenge alone	8/10	-	5/5	5/5
Ether 2 days before challenge	N.T. ^d	N.T.	5/5	5/5
C.A.A. 2 days before challenge	N.T.	N.T.	4/5	5/5

^a c.f.u. of challenge of Expt 1 = 7×10^4 ; Expt 2 = 1.1×10^5 ; and Expt 3 = 1.0×10^5

^b Complete contamination prevented lung culture results of Expt 1

^c The statistical significance when compared to the infection alone group by the 2x2 contingency tables of Finney et al (1963).

^d NS = Not significant; * = significant at the 5% level; ** = significant at the 1% level.

N.T. = Not tested.

given 24 h before challenge did not significantly reduce either lung pathology or culture. No passive protective activity was demonstrated by lung pathology or culture by either 1/2 or 1/20 dilution of pooled convalescent TBW mixed with challenge. This was peculiar since these dilutions were previously protective.

Pooled convalescent serum was passively protective given intranasally 48 h before challenge and prevented the development of pathology and culture as efficiently as when it was given as a mixture. Pooled convalescent TBW was able to passively protect by preventing pathology and culture on one experiment if given intranasally, 48 h before challenge. However the inconsistency of the pooled convalescent TBW might reflect the lower potency compared to pooled convalescent serum. The overall conclusion was that convalescent serum and TBW could be given intranasally separately from the challenge and up to 48 h beforehand and still exhibit impressive protective activity.

2.12 Ability of pooled sera and TBW to neutralise the HSF activity of pertussis vaccine

As yet there has been no report on the development of antibodies to the biological activities associated with pertussigen after a murine pulmonary infection. It seemed relevant to establish if an immune response to pertussigen took place during such an infection since what may be taken as this component (under a different name) has been proposed by Sato et al (1980) as the protective antigen in both the IC and SLIN murine infections.

Pooled sera and TBW were therefore examined for ability to

neutralize the HSF activity of B. pertussis vaccine. To ensure maximum chance of detecting antibodies, low doses of vaccine, 1.5 and 3 HSD₅₀, were mixed with pooled undiluted convalescent sera and TBW. The mixtures were injected, after 18 h incubation at 4°C, into 7 wk old mice and the induction of histamine sensitivity tested by histamine challenge 5 days later.

As shown in Table 23 pooled convalescent sera did not neutralize 3.0 HSD₅₀ of pertussis vaccine, since between 5 to 7 out of 7 mice became sensitive to histamine. In the vaccine-alone group, 7 out of 7 mice died when the lower vaccine dose of 1.5 HSD₅₀ of pertussis vaccine was used, day 49 and day 61 pooled convalescent sera reduced the number of deaths (2 out of 7) and (5 out of 14) respectively. However these were not significantly different compared to vaccine alone when analysed by 2 x 2 contingency tables of Finney et al (1963). The U.S. Standard Antipertussis serum did however significantly reduce histamine sensitivity with no mice dying after histamine challenge.

Pooled normal and acute phase sera were unable to neutralise either 1.5 or 3 HSD₅₀ of pertussis vaccine: there were 7 out of 7 mice dead for both sera mixed with either dose of vaccine.

Pooled convalescent TBW was unable to neutralise 3.0 HSD₅₀ for 6 or 7 out of 7 mice were still sensitive to histamine compared to 7 out of 7 sensitive in the vaccine alone group (Table 24). They were still unable to neutralize 1.5 HSD₅₀ from the observation that 5 or 7 out of 7 and 10 or 14 out of 14 sensitive to histamine compared with 5 out of 7 sensitive in the vaccine alone group.

Pooled normal TBW was unable to neutralise 1.5 or 3 HSD₅₀

Table 23 : Ability of pooled sera from normal and B. pertussis infected mice sampled at different stages after instillation of a pulmonary infection to neutralise HSF in pertussis vaccine. The U.S. Standard Antipertussis serum was used as a reference

Expt No.	Titration of HSD ₅₀ (o.u.)	U.S. Standard anti-pertussis	Histamine sensitivity of mice given 1.3 o.u. pertussis vaccine (No. Responding)											
			(Type of mouse sera sampled (days post-instillation sampled))											
	1.3	0.65	0.33	pertussis	N ^b	N	I (15)	I (34)	I (40)	I (42)	I (47)	I (48)	I (49)	I (61)
1	7/7	5/7	0/7	0/7	N.T. ^c	7/7	6/7	6/7	7/7	5/7	7/7	7/7	7/7	N.T.
2	5/7	2/7	0/7	N.T.	7/7	7/7	6/7	7/7	7/7	6/7	7/7	4/7	2/7	13/14

a = Mice were given the vaccine/pooled serum mixture i.p.

b = N = Normal; I = Infected.

c = N.T. = Not tested.

Table 24 : Ability of pooled TBW from normal and B. pertussis infected mice sampled at different stages after instillation of a pulmonary infection to neutralise HSF in pertussis vaccine. The U.S. Standard Antipertussis Serum was used as a reference.

Expt No.	Titration of HSD ₅₀ (o.u.)	U.S. Standard Anti-pertussis N ^b	Histamine sensitivity of mice given 1.3 o.u. pertussis vaccine (No. Responding) (No. Challenged)											
			(Type of mouse TBW sampled (days post-installation sampled)											
			I	I	I	I	I	I	I	I	I	I	I	I
1	7/7	5/7	0/7	0/7	6/7 ^c	N.T.	7/7	6/7	7/7	7/7	7/7	6/7	6/7	N.T.
2	5/7	2/7	0/7	0/7	6/7	6/7	6/7	7/7	7/7	7/7	5/7	6/7	6/7	14/14
														10/14

a Mice were given the vaccine/pooled TBW mixture i.p.

b N = Normal; I = Infected.

c N.T. = Not tested.

pertussis vaccine since 6 out of 7 mice died given either dose of pertussis vaccine. Pooled acute phase TBW was also unable to neutralize HSF since 7 out of 7 mice died at both doses of vaccine.

Pooled day 49 and day 61 convalescent sera were the only pooled samples which were able to reduce the histamine sensitising activity of pertussis vaccine. However the difference between these and the vaccine alone was not significant when analysed by the 2 x 2 contingency tables. Only U.S. Standard Antipertussis Serum significantly reduced histamine sensitivity.

SECTION III: HSF AND LPF RESPONSIVENESS OF CONVALESCENT ANIMALS

The foregoing showed that convalescent sera and TBW were not detectably able to neutralise histamine-sensitizing activity of pertussis vaccine. However, there remained the possibility that the fully-recovered mice themselves might be somewhat resistant to induction of histamine sensitization as tested by injection of vaccine and subsequent challenge. This was therefore done at 53 and 61 days post-instillation. The same animals were also tested for possible refractoriness to induction of leucocytosis by the vaccine. Thus on the 4th day after ip. administration of the vaccine the animals were blood-sampled for leucocyte count and then on the 5th day histamine challenged.

3.1 Leucocyte count in vaccinated normal and convalescent mice

Some of the mice which had been infected to raise day 49

convalescent sera and TBW in Experiment 3 were used to see if they were unresponsive to the LPF in 1.33 and 4.00 o.u. pertussis vaccine given i.p. (Table 25). The mean leucocyte count on day 53 convalescent mice receiving no vaccine (6456 mm^{-3}) was similar to that of normal unvaccinated mice (6767 mm^{-3}). However no reduction in the mean leucocyte count in day 53 convalescent mice was found at either vaccine dose. Of mice given 1.33 o.u. vaccine day 53 convalescent mice had a mean leucocyte count of 10790 mm^{-3} compared to $11,324 \text{ mm}^{-3}$ in normal mice. No difference was found in the mean leucocyte count of mice treated with 4 o.u. pertussis vaccine with day 53 convalescent mice having $16,710 \text{ mm}^{-3}$ normal mice having a count of 15136 mm^{-3} .

The next experiments into the possible neutralisation of LPF were done on day 65 convalescent mice reserved from sampling experiments 4 to 7. Four groups of day 65 convalescent mice were investigated independently using low doses of vaccine to ensure maximum chance of detecting neutralisation. These 4 experiments were totalled and analysed by comparing the mean leucocyte count of convalescent and normal mice at each vaccine dose by the t-test on the geometric mean.

Again the mean leucocyte count (mm^{-3}) of day 66 convalescent (9044) and normal mice (9115) receiving no vaccine were not significantly different. In mice given 0.44 o.u. pertussis vaccine the mean leucocyte count of convalescent mice ($10,767 \text{ mm}^{-3}$) less than normal mice ($12,957 \text{ mm}^{-3}$) but the difference was not significant by the t-test. Of mice given 1.33 o.u. pertussis vaccine the mean leucocyte count of day 65 convalescent mice ($11,290 \text{ mm}^{-3}$) was significantly less

Table 25 : Leucocyte count in normal and B. pertussis convalescent mice given various doses of B. pertussis vaccine

Days post infection (Expt. group No.)	No. of mice	Leucocyte count ml ⁻¹ (G.M. (\pm SD) in mice treated as follows									
		Dose of vaccine (o.u. ml) per mouse									
		N ^a	I ^a	N	I	I	N	I	N	I	I
53(E3)	N ^a =7 I ^a =10	6767 (4839-9462)	6456 (4058-10270)	N.T. ^a	N.T.	0.44	11324 (7407-17310)	10990 (6492-18603)	15136 (11665-19638)	16710 (11376-24547)	4.00
65(E4)	N=7 I=10	7943 (7834-8053)	8298 (7638-9015)	10232 (10115-10351)	11066 (10423-11748)		18365 (18071-18663)	13335 (13212-13458)	N.T.	N.T.	
65(E5)	N=7 I=10	11481 (11402-11561)	8709 (8649-8770)	21928 (21627-22233)	13076 (12147-14076)		25468 (24603-26363)	12560 (11220-14060)	N.T.	N.T.	
65(E6)	N=20 I=20	7898 (4550-13670)	10003 (5933-16988)	15684 (12163-20224)	12689 (10228-15755)		14687 (10972-19629)	11187 (7476-17127)	N.T.	N.T.	
65(E7)	N=20 I=20	9081 (7143-11544)	8536 (5384-13533)	10658 (7932-14319)	9239 (7259-11760)		18469 (12804-26640)	13448 (11048-16368)	N.T.	N.T.	
Mean of E4-7	N=61 I=70	9115 (5633-14750)	9044(NS) (5698-14356)	12957 (8179-20527)	10767(NS) (7064-16411)		17488 (12315-24833)	11290* (7136-17862)			

a N = Normal; I = Infected; N.T. = Not tested

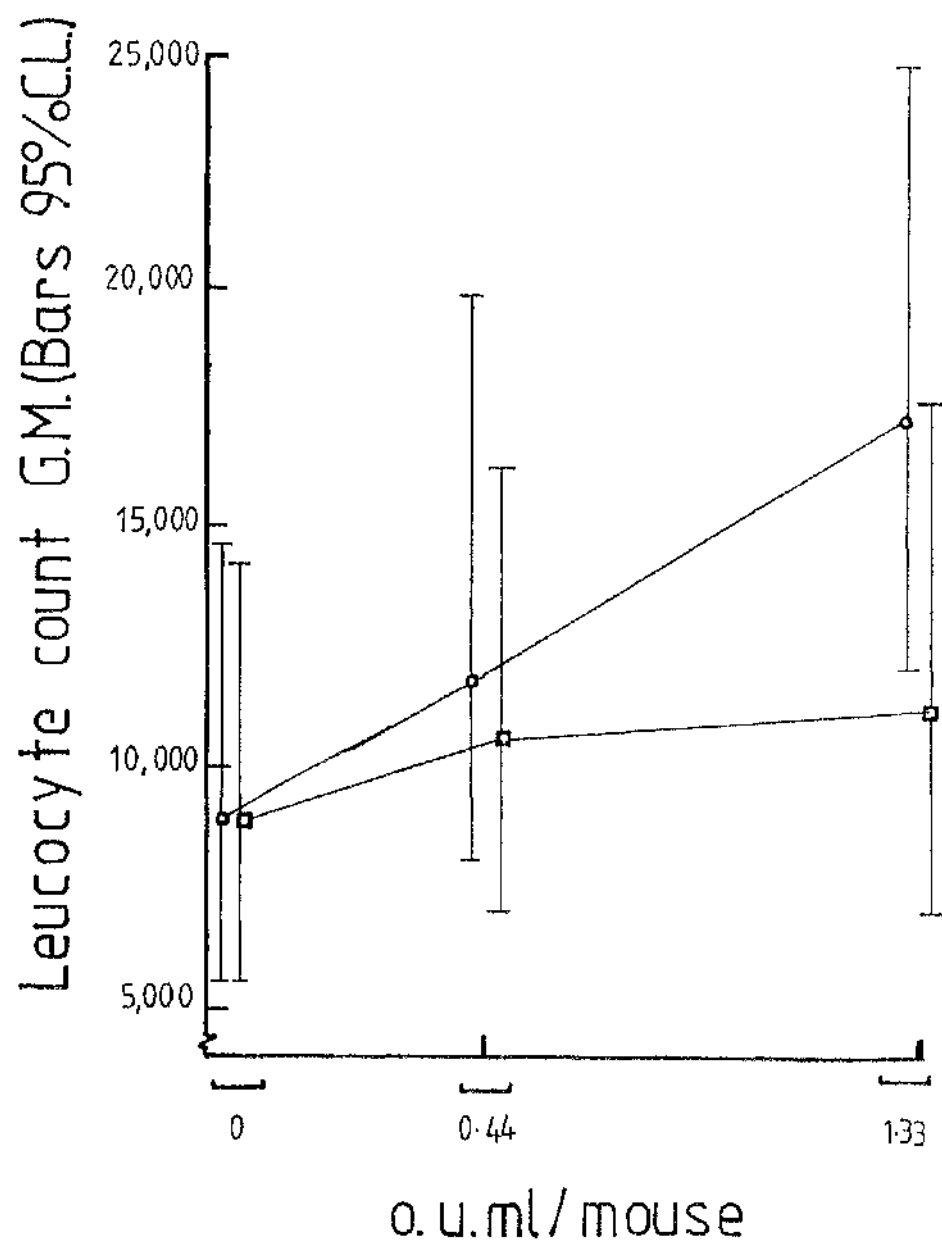
b The statistical significance between the normal and infected mice at each vaccine dose by the t-test.

NS = Not significant; * = significant at the 5% level.

Figure 13. Leucocyte count in the normal and B. pertussis day 65 convalescent mice given various doses of B. pertussis vaccine. The geometric mean (\pm 1 S.D.) are given. The numbers in parenthesis are the number of mice tested.

○—○ Normal control mice

□—□ Convalescent mice.



than that of normal mice ($17,488 \text{ mm}^{-3}$).

Figure 13 shows the mean leucocyte count of normal and day 63 convalescent mice in E4-7 plotted against vaccine dose. Normal mice gave a good dose-response curve to 0.44 and 1.33 o.u. ml B. pertussis vaccine. However day 65 convalescent mice gave a flattened dose-response curve with both 0.44 and 1.33 o.u. ml B. pertussis vaccine having had the leucocyte promoting activity neutralised.

3.2 Histamine sensitivity in vaccinated normal and convalescent mice

On the day after the leucocyte counts of the above mice were recorded, their histamine sensitivity was determined by i.p. challenge with histamine (Table 26). No reduction in refractoriness towards histamine sensitization was found in day 54 convalescent mice given either 1.33 o.u. or 4.00 o.u. pertussis vaccine. Of mice given 1.33 o.u., 7 out of 10 convalescent mice were still sensitive to histamine compared to 7 out of 7 normal mice. Four o.u. pertussis vaccine sensitized 10 out of 11 convalescent mice and 7 out of 10 normal mice. However the convalescent mice without pre-treatment with vaccine were somewhat sensitive to histamine, presumably as a result of the pulmonary infection with 7 out of 12 convalescent mice histamine sensitive.

These results indicate that the histamine sensitising effect of a murine pulmonary infection of B. pertussis is longer lasting than that of leucocytosis promoting effect.

Histamine sensitization results of day 66 convalescent mice of Experiments 4-7 were totalled and analysed by the 2×2 contingency

Table 26 : Histamine sensitivity in normal and B. pertussis convalescent mice given various doses of B. pertussis vaccine

Day of infection (Expt.No.)	Histamine sensitivity ($\frac{\text{Death}}{\text{Total}}$)									
	Dose of vaccine (o.u. ml) per mouse									
	N ^a	I ^a	N	I	N	I	N	I	N	I
54(E3)	0/6	7/12	N.T. ^a	N.T.	7/7	7/10	7/7	7/10	7/7	10/11
66(E4)	0/10	0/7	0/7	2/12	5/7	9/14	N.T.	9/14	N.T.	N.T.
66(E5)	0/6	1/10	3/7	3/10	6/7	10/10	N.T.	10/10	N.T.	N.T.
66(E6)	1/20	1/20	18/20	10/20	20/20	7/18	N.T.	7/18	N.T.	N.T.
66(E7)	0/20	1/20	9/20	3/20	19/20	15/20	N.T.	15/20	N.T.	N.T.
Totals E4-7	1/56	3/57 (NS)	30/54	18/62*	50/54	41/52 (NS)				

a N = Normal; I = Infected and NT = Not tested.

b The statistical significance between normal and infected mice at each vaccine dose by the 2 x 2 contingency tables of Finney et al (1963).

NS = Not significant; * = significant at the 5% level.

tables of Finney et al (1963) by comparing the histamine sensitivity of the convalescent and normal mice of the same age and sex at each vaccine dose. Histamine sensitivity of day 66 convalescent mice had returned to normal, only 3 out of 57 showing sensitivity compared to 1 out of 56 sensitive in the normal mice. In mice given 0.44 o.u. pertussis vaccine day 66 convalescent mice were significantly less sensitive to histamine (18 out of 62) than normal mice (30 out of 54). Mice given 1.33 o.u. pertussis vaccine day 66 convalescent mice were less sensitive (41 out of 52) than normal mice (50 out of 54) but the difference between the two groups was not significant by the 2 x 2 contingency tables.

The neutralisation of leucocyte promoting activity is more pronounced at the higher dose of vaccine (1.33 o.u.) which is the opposite result found in the neutralisation of histamine sensitizing activity where neutralisation was more pronounced at the lower dose (0.44 o.u.). This might be indicative of the sensitivity of the assays, ie it might require less pertussigen to induce histamine sensitivity than leucocytosis.

SECTION IV: NATURE OF IMMUNOGLOBULINS IN CONVALESCENT TBW AND SERA

Geller and Pittman (1973) examined the development of B. pertussis specific immunoglobulins in sera and TBW after a sublethal murine pulmonary infection. However the target antigens of the developing Igs was not investigated. In the present study, IgM, IgG, IgA and IgE were removed from pooled convalescent sera and TBW after

which agglutination and passive protective activity were assayed.

Pooled day 61 convalescent sera was used since it had the highest SLIN passive protective activity and the agglutinin response of all pooled convalescent sera were similar. Pooled day 48 convalescent TBW was used because of availability. It was concentrated x10 to increase SLIN passive protective activity.

Details of the procedures used to remove the immunoglobulins are given in Materials and Methods.

4.5 Agglutination tests on pooled day 61 convalescent sera and 10x concentrated pooled day 48 convalescent TBW either untreated or with Ig classes selectively removed

Since agglutination tests were one of the main serological assays in analysing convalescent serum in this study, it was decided to determine if the agglutinins at day 61 of infection came from a particular class of immunoglobulin (Table 27).

Untreated pooled day 61 convalescent sera was tested at a 1/5 dilution since this was the dilution of sera at which immunoglobulins were selectively removed. Untreated pooled normal sera was also titrated at this dilution.

Untreated pooled day 61 convalescent sera has a reciprocal titre of 8 for both agglutinating strains of B. pertussis 18-323 and 18-334. The removal of IgG from pooled day 61 convalescent sera completely removed the agglutinins for both 18-323 and 18-334 strains of B. pertussis, whereas the removal of IgM, IgA and IgE from pooled day 61 convalescent sera did little to change the reciprocal

Table 27 : Reciprocal agglutination titre on pooled day 61 convalescent sera and the same sera with the immunoglobulin classes IgM, IgG, IgA and IgE selectively removed. All sera were at a 1/5 dilution and agglutinins against both B. pertussis 18-323 and 18-334 strains were investigated

Treatment of Day 61 sera (1/5 dln)	Reciprocal titre ^a	
	18-323	18-334
None	8	8
IgM removed	8	4
IgG removed	-	-
IgA removed	8	4
IgE removed	8	4
Untreated pooled normal sera (1/5 Dln)	-	-

a The reciprocal titres shown were obtained on two independent occasions.

agglutination titre with the 3 sera having reciprocal titres of 8 and 4 for B. pertussis 18-323 and 18-334 strains respectively. Untreated pooled normal sera at 1/5 dilution did not agglutinate either strain. The major agglutinating immunoglobulin in sera at day 61 of a pulmonary infection was therefore IgG.

Concentrated pooled convalescent TBW without any Ig class removed still gave no agglutination with either B. pertussis strain as did the unconcentrated pooled TBW. So removal of IgG classes had no observable effect on the agglutinin titre.

4.6 Sublethal intranasal passive protection tests on pooled day 61 convalescent sera and 10x concentrated pooled day 48 convalescent TBW either untreated or with Ig classes selectively removed

The class(es) of immunoglobulins responsible for the SLIN passive protective activity found in both pooled convalescent sera and TBW was investigated to see which immunoglobulin(s) contributed to SLIN passive protective activity in pooled convalescent sera and TBW. However although it was pooled day 61 convalescent sera and 10x concentrated pooled day 48 TBW that were used, the passive protective Ig(s) on these days might be completely different to those at day 34 post-instillation.

Sublethal intranasal passive protective activity of pooled convalescent sera and TBW, either untreated or with Ig classes selectively removed, was titrated twice and the $PD_{50} \text{ ml}^{-1}$ and 95% C.L. calculated for both the lung pathology and culture results from the cumulative totals of both titrations (Table 28). Untreated pooled convalescent serum and TBW were the reference standard from which the

Table 28 : Sublethal intranasal passive protective activity of pooled day 61 convalescent sera and the same sera with the immunoglobulin classes IgM, IgG, IgA and IgE selectively removed and assessed by lung pathology and culture

Treatment of sera	Passive protective activity as determined by	
	Lung pathology $PD_{50} \text{ ml}^{-1}$ (95% CL)	Lung culture $PD_{50} \text{ ml}^{-1}$ (95% CL)
Untreated	20,000	13,320
IgM removed	20,000 (8,680-40,000)	20,000 (8,000-133,320)
IgG removed	8,000 (3,060-20,000)	10,000 (3,620-26,660)
IgA removed	8,000 (4,000-25,000)	10,000 (3,620-26,660)
IgE removed	13,320 (8,000-57,140)	10,000 (3,320-25,000)

95% C.L. were calculated. The proportion of significant scores for both lung pathology and culture in the titrations are tabulated in the Appendix 17 (pooled serum) and Appendix 18 (pooled 10x concentrated TBW).

The $PD_{50} \text{ ml}^{-1}$ of untreated serum was 20,000 as determined by lung pathology results and 13,320 by lung culture results (Table 27). This correlates with previous titrations of this pooled serum. Pooled day 61 convalescent serum with IgM removed also had a $PD_{50} \text{ ml}^{-1}$ of 20,000 determined by both lung pathology and culture results. It would seem that the IgM in this serum was not involved in SLIN/mix passive protective activity.

The removal of IgG reduced the SLIN passive protective activity significantly when determined by lung pathology results with $PD_{50} \text{ ml}^{-1} = 8,000$ and upper confidence limit of 20,000. However the reduction was not significant when determined by lung culture results where $PD_{50} \text{ ml}^{-1}$ was 10,000 but the upper confidence limit of 26,660 was greater than the $PD_{50} \text{ ml}^{-1}$ of untreated serum.

Although IgA removal produced a reduction in $PD_{50} \text{ ml}^{-1}$ by both lung pathology (8,000) and lung culture (10,000), the upper confidence limits of both the lung pathology (25,000) and lung culture (26,660) were both greater than the $PD_{50} \text{ ml}^{-1}$ of untreated serum. The reduction was therefore not significant.

A similar result was found with IgE removal in that the $PD_{50} \text{ ml}^{-1}$ by both lung pathology (13,320) and lung culture (10,000) were reduced, the upper confidence limits (57,140 and 25,000) respectively were both greater than the mean of untreated serum.

Therefore although removal of IgG, IgA or IgE each reduced SLIN passive protective activity, IgG lung pathology results were the only case where the difference was significant.

When a similar investigation was done on 10x concentrated pooled day 48 TBW, the untreated fluid had a $PD_{50} \text{ ml}^{-1}$ of 1320 by both lung pathology and lung culture results. Titration of the $PD_{50} \text{ ml}^{-1}$ of this TBW with IgM removed produced dissimilar results by lung pathology and lung culture. The $PD_{50} \text{ ml}^{-1}$ (and upper confidence limit) determined by lung pathology was 1000 (66,600) and by lung culture was 200 (not defined). Therefore by lung pathology results IgM seemed to have no involvement in SLIN passive protective activity, but by the culture results it appeared as if it was involved. However unlike the pathology results, which were based on two experiments the culture scores were only determined once, and it would be difficult to draw conclusions without repeating.

IgG removal from convalescent TBW significantly reduced the $PD_{50} \text{ ml}^{-1}$ of TBW to 60 by lung pathology results since the upper confidence limit was 320. The $PD_{50} \text{ ml}^{-1}$ for the lung culture results was also reduced to 38 but the upper confidence limits were undefined. However although the $PD_{50} \text{ ml}^{-1}$ of TBW with IgA removed (580) and IgE removed (500), the reduction was not significant since the upper confidence limits (4,000 and 2,840 respectively) overlapped with the $PD_{50} \text{ ml}^{-1}$ of untreated TBW. The lung culture results did not allow the $PD_{50} \text{ ml}^{-1}$ to be determined for either the titration of TBW with IgA or IgE removed.

IgG seems to have a more marked involvement in the SLIN

Table 29 : Sublethal intranasal passive protective activity of 10x concentrated pooled day 48 convalescent TBW and the same TBW with the immunoglobulin classes IgM, IgG, IgA and IgE selectively removed and assessed by lung pathology and culture

Treatment of TBW	Passive protective activity as determined by	
	Lung pathology	Lung culture
	PD ₅₀ ml ⁻¹ (95% CL)	PD ₅₀ ml ⁻¹ (95% CL)
Untreated	1320	1320
IgM removed	1000 (160 66,660)	200 (N.D. ^a)
IgG removed	60 (N.D., 320)	38 (N.D.)
IgA removed	580 (60 4000)	N.D.
IgE removed	500 (40 2840)	N.D.

a N.D. = Non defined since computer was unable to calculate this value due to poor data.

passive protective activity of TBW than in serum. Although in both TBW and serum it is the only immunoglobulin which when removed leads to a significant reduction in this activity. IgA and IgE appear to have a less significant degree of involvement in SLIN passive protective activity of serum and TBW. IgM plays no demonstrable role in the serum activity and the question of its role in SLIN passive protective activity remains unanswered.

DISCUSSION

SECTION I PATHOPHYSIOLOGY OF *B. PERTUSSIS* INFECTIONS IN THE HUMAN
AND OTHER SPECIES

Several important aspects of the disease process in pertussis, such as the basis of the paroxysmal cough, are still unexplained. However since direct experiments on humans are not possible, many attempts have been made to establish animal models which would mimic the main features of the disease. So far, no fully satisfactory model has been demonstrated although the one described in this thesis is believed to have many worthwhile features.

Primates, of which the Taiwan monkey (*Macaca cyclopsis*) has emerged as the best species for production of paroxysmal cough, have been proposed as animal model of pertussis (Lin, 1958; Huang et al, 1962; Stanbridge and Preston, 1974). However, reports tend to be few and not widely confirmed. Nevertheless the Taiwan monkey, when infected with *B. pertussis* developed a disease with several pathophysiological features similar to human pertussis: in particular, the incubation period, time-course, leucocytosis and duration of paroxysmal coughing were similar. So are the immunological responses of opsonocytophagic index and induction of active immunity by vaccination or by infection and recovery (Lin, 1958; Huang et al, 1962).

The marmoset species which exhibits serotype shift during infection, as in human pertussis, but does not cough, has been much studied by Stanbridge and Preston (1974). These authors have focused attention on responses to the agglutinogens, largely to the exclusion of other pathophysiologically or immunologically significant components

of the bacillus. From a practical viewpoint however, primates are too rare and expensive for widespread adoption as animal models of pertussis, since such a model, ideally would be sufficiently available to permit the routine potency and safety testing of vaccines.

Various other animal species have been investigated (dogs, ferrets, rats) but none, apart from the mouse, in any detailed fashion. Thus while the rabbit can undergo respiratory tract infection with B. pertussis, it does not cough, although serotype shift during the infection has been noted (Preston, 1980). This leads to consideration of the mouse as the prime candidate for a pertussis model, and one of the main objects of the present work was to explore further the suitability of this species.

There have been extensive studies on three main types of experimental B. pertussis infection in the mouse, notably (a) the intracerebral infection which is used as part of the standard mouse protection test for vaccine potency but is highly artificial; (b) the lethal intranasal infection, which kills mice within 1-2 weeks through overwhelming growth of the bacteria in the lungs; and (c) the sublethal intranasal infection which has been studied here. This last-mentioned has a similar time-course of physiological and immunological changes to those in human pertussis and also is prevented by a heat-labile antigen of the bacillus (Fisher, 1955). Indeed it has been proposed by Pittman et al (1980) as a worthwhile animal model for studying the disease. The main imperfection of the intranasally sub-lethally infected mouse is that it does not appear to cough; on this score however, detailed enquiry has failed to uncover any agent which induces coughing in mice (Wardlaw, personal comm.) and it may be that the cough reflex is absent in this species.

The positive features of this mouse model are highlighted in Table 29 which makes comparisons with human pertussis. It will be noted that the mouse model resembles the human disease in 8 out of 10 listed characteristics. Both infections have age-related severity. This was established in mice very soon after this species was subject to respiratory tract infection (Culotta et al, 1938) and confirmed in later studies, eg Standfast (1951) and Pittman et al (1980). The age-related severity of pertussis in man has been amply documented and further reinforced by experience in recent epidemics in the United Kingdom (Miller et al, 1982).

Mouse lung lesions (Burnet and Timmins, 1937) after SLIN infection with B. pertussis show several similarities in pathology with the human disease (Mallory and Horner, 1912). Multiplication of the organisms was localized in the mucus lining the bronchial tubes; there was degeneration of cilia-bearing epithelial cells and interstitial pneumonia.

Adhesion has been proposed as the first step in the pathogenesis of pertussis (Olson, 1975; Pittman, 1979) but is obviously difficult to study directly in humans. However various experimental systems for studying adhesion of B. pertussis have been developed (Holt, 1972; et al, Muse, 1977; Burns, 1982). The last-mentioned author, who worked in this department, developed an adhesion assay in which B. pertussis fresh isolates which were IN virulent, were shown to be more adhesive to mouse lungs than were C-mode or Phase IV strains. This observation of a relation between adherence and virulence should now be more extensively studied to determine its general validity.

The challenge doses used to establish a murine sublethal

Table 30 : Comparison of the murine sublethal pulmonary infection of
B. pertussis and human whooping cough

Characteristics	Murine sublethal Pulmonary Infection	Human Whooping Cough
Age-related severity	+	+
Lack of invasiveness	+	+
Multiplication and organism disappearance around Day 30	+	+
Histamine sensitization	+	+
Leucocytosis or lymphocytes	+	+
Hypoglycaemia	+	+
Hyperinsulinaemia	+	NI ^a
Attenuation of adrenaline- mediated hyperglycaemia	+	+
Paroxysmal cough	-	+
Transmission between individuals in close contact	-	+

a NI = Not Investigated.

However pertussis vaccine produces a transient hyperinsulinaemia in
the child. (Hannik and Cohen, 1979).

pulmonary infection have been between 2×10^3 cfu B. pertussis (Andersen, 1953b; Dolby et al, 1961) and 3×10^5 cfu B. pertussis (Pittman et al, 1980). These are between 10 and 2000 times greater than the dose used by Macdonald and Macdonald (1933) to infect successfully two of their unimmunised children. In 7-day old mice Pittman et al (1980) found the LD₅₀ was 1000 times less than in 3-4 wk old animals.

In the present investigation, the infection peak of murine sublethal pulmonary infection occurred between days 11 to 14 post-instillation. At the peak of infection, around 10^7 to 10^8 cfu B. pertussis could be recovered from the lungs of individual mice. Thereafter the count declined but viable bacteria could still be recovered on day 31 and 35 when these experiments were terminated. This time-course pattern is similar to that reported by other investigators using other strains of B. pertussis and other breeds of mice (Proom, 1947; Andersen, 1953b; Dolby et al, 1961). The last-mentioned authors emphasised that the number of bacteria in the SLIN dose should not exceed 10^7 cfu otherwise a lethal infection was likely. Proom (1947) recovered B. pertussis from the lungs as late as 56 days after SLIN infection and Andersen (1953b) as late as the 59th day post-instillation. These times are much longer than those found in man, but it must be remembered that isolation of B. pertussis from the human is not done by culturing portions of lung tissue. Indeed the possibility can not be dismissed that the bacteria may persist in the human lung for some time after they can be recovered from cough plates or nasopharyngeal swabs (Olson, 1975).

The gross pathological changes which occurred during the murine

sublethal pulmonary infection also peaked between days 14 and 18 and had scarcely declined at day 35, by which time most of the live B. pertussis had been eliminated. This indicated that the clearance mechanisms for removing live bacteria were more rapidly acting than those involved in resolving the damage caused by the infection. Comparable data for man are unavailable for obvious reasons.

Leucocytosis, hypoglycaemia and histamine sensitivity, which are pathophysiological features of both human pertussis and murine infection, persist until at least day 33 in the latter (Pittman et al, 1980) and until well into the paroxysmal stage in the former (Lagergren, 1963; Regan and Tolstouhrov, 1936; Sanyal, 1960). Therefore the mouse model supports the hypothesis of Pittman (1979) that pertussis should be regarded as an exotoxin disease in which a non-invasive bacillus excretes a toxin with long-lasting effects - in this case the diverse pharmacological alterations produced by pertussigen.

As already emphasised, the major difference between human pertussis and the murine sublethal pulmonary infection is that the mice do not appear to cough. However, Pittman (1951) suggested that there was some significance in the parallelism between the time course of histamine sensitivity during the murine infection and the duration of the paroxysmal coughing in the child. This led to the further proposal that there might be similar underlying pharmacological reactions taking place but with different outward manifestations in humans and mice. This might be analogous to the species differences in systemic anaphylaxis where the target organs differ in different

species, eg the lungs in man and guinea pig and the liver in the dog.

Lack of serotype shift in the murine infection was considered by Carter and Preston (1981) to detract from its value as a pertussis model. However in the studies which they reported, a majority of their mice died during the infection which could hardly therefore be described as 'sublethal'. Contrary to Carter and Preston's conclusions, their actual data show that the few surviving mice did actually exhibit serotype shift of the infecting B. pertussis from type 1 to type 1,3.

Pertussis is far from being alone in lacking a perfect animal model, for relatively few bacterial respiratory tract infections of man have a fully satisfactory model in a convenient laboratory species. For example, experimental rat pulmonary Haemophilus influenzae infection leads to bacteraemia and meningitis; however a lower incidence of meningitis is found in bacteraemic rats than in infants (Moxon et al, 1974). Pseudomonas aeruginosa, an opportunistic pathogen in cystic fibrosis, will only grow in the respiratory tract of rats or other experimental animals when instilled with an agar plug (Cash et al, 1979). The pulmonary infection of the Syrian hamster with Mycoplasma pneumoniae does, however, seem to be fairly satisfactory as a model, in that the organisms remain localized in the ciliated epithelium of the upper respiratory tract, as in man, and similar pathological changes occur in both species (Dajani et al, 1965).

SECTION II IMMUNE RESPONSES TO B. PERTUSSIS INFECTIONS IN MOUSE AND MAN

2.1 Protection against infection

The high incidence of severe pertussis in infants, particularly those below 6 months of age, has been attributed to the lack of transfer of maternal protection (J.C.V.I., 1977). However Preston et al (1974) found some infants had type 1 and type 2 but not type 3 agglutinins at low levels, which could be explained by the half-life of the IgG of maternal origin. He suggested that maternal antibodies might be transferred but that they might not be directed against important virulence factors, notably agglutinin 3. Agglutinin 3 might be absent from the mother if her infection had been a long time in the past when the 1,2 serotype was prevalent.

In an early report, protection of children against B. pertussis infection by passive immunization was done with convalescent children's serum (Debre, 1923) and hyperimmune serum (McGuinness et al, 1944). This appeared to be successful if done during the incubation period; however higher doses of hyperimmune serum were necessary to demonstrate the therapeutic value of immune serum and it is not now used in the U.K. although it still finds favour in continental Europe (F. Sheffield, per. comm.). Convalescent children's serum was active in mice. When given by either i.p. or IN route it protected mice from both lethal (Winter, 1953) and sublethal pulmonary infections (North et al, 1939). The same is true of mouse convalescent serum taken from animals that had recovered from SLIN infection (North, 1946; this investigation). Normal children's and normal mouse sera were non-protective in these systems (Winter, 1953; North et al, 1939).

The present investigation not only confirmed the development of SLIN passive protective activity in serum during a murine sublethal pulmonary infection but also, apparently for the first time, showed that

such activity could also be demonstrated in tracheobronchial washings (TBW). Both pooled sera and TBW from sublethally infected mice had passive protective activity by day 34 post-instillation and their activity had increased 10 fold by day 61. The ratio of passive protective activity between mouse convalescent sera to mouse convalescent TBW was usually approx 50:1 which is the same as the protein ratio of the convalescent sera and TBW. Normal and acute-phase (15-day) sera and TBW had no such activity. The U.S. Standard Antipertussis serum was at least 20 fold more active than day 61 mouse convalescent sera.

An attempt was made to determine the nature of the bacterial components against which the passively protective antibodies in mouse sera were directed. To be definitive, this would have to be done either with monoclonal antibodies administered passively or with purified virulence factors which could be used as absorbents for the convalescent sera. Since neither was available, the effect of absorbing the convalescent sera with different virulence types of B. pertussis cells was done. Phase I cells were shown to absorb the passive protective activity of convalescent serum completely, whereas Phase IV B. pertussis did not. This indicated that the antigens against which the passively protective antibodies were directed were present on the surface of Phase I B. pertussis but not on the surface of Phase IV.

Andersen (1953b) investigated the occurrence of active SLIN immunity by infecting mice by this route 70 days after a previous pulmonary infection with B. pertussis from which the mice had by then recovered. She found that the convalescent mice were able to clear

B. pertussis from the lungs 4-8 days after re-infection which was much faster than during the primary infection. However since no normal controls of old mice were used, this might have been due to the age effect associated with B. pertussis infections in mice (Standfast, 1951) rather than being due to acquired immunity.

In the child, it is generally accepted that recovery from pertussis confers solid immunity of at least several years duration. (Pittman, 1970).

Active immunization against the human pulmonary infection reduces the H.E.A.R. by between 65-80%. This parameter correlates strongly with the vaccine potency as measured by the mouse protection test where IC challenge was administered to mice 14 days after i.p. vaccination. However comparison between human and murine vaccine efficacy against pulmonary challenges is difficult. Mice at 5 weeks of age, ie 2 weeks after i.p. vaccination, are much less susceptible to a pulmonary challenge due apparently to an age associated resistance to pulmonary infections (Standfast, 1951; Pittman et al, 1980).

Turning now to the IC infection of mice with B. pertussis, no development of antibodies in sera from children between 1-12 wk after infection was found by Dolby and Stephens (1973); however in 3 uninfected children, exposed to active cases 2 out of 3 developed mouse IC passive protective activity. Perhaps the exposure provided a booster stimulus to pre-existing priming immunity.

Cooper (1952) found mice after day 30 of a sublethal pulmonary infection were able to resist IC challenges of between 1-5 LD₅₀. However, until the present study, the development of IC passive protective activity during a murine sublethal pulmonary infection has not been investigated. The results were clear cut: the convalescent

sera and TBW from the sublethal intranasally-infected mice had no protective activity against either 1 or 100 LD₅₀ of strain 18-323 given intracerebrally in a "mix" test. The U.S. Standard Anti-pertussis serum was however passively protective by this challenge route but 300 fold less active than by the SLIN/mix test. However if the same ratio between potency of the latter and mouse convalescent serum exists in the IC challenge as exists in the SLIN challenge, any IC passive protective activity present in mouse convalescent serum would remain undetected.

The existence of two distinct protective antigens by the IC and IN infection routes was demonstrated by Dolby and Standfast (1961). The "IC antigen" was heat-labile (100°C) and the "IN antigen" heat-stable (100°C). However, it should be noted that the "IN antigen" refers to the lethal IN infection. Fisher's earlier work was in agreement with these findings but made the further significant observation that the protective antigen in the SLIN test was heat-labile (100°C) and thus different from the protective antigen in the lethal IN system.

2.2 Serology

Human convalescent sera has long been known to contain B. pertussis agglutinins (Bordet and Gengou, 1906). However 99% of sera from healthy adults and non-vaccinated, non-infected infants of 1-6 months also contain low agglutinin titre to B. pertussis (Dolby and Stephens, 1973). The authors found this agglutinin was removed from all baby sera by absorption with B. pertussis strain which had

only agglutininogen 1 and from 76 out of 93 adult sera by absorption with agglutininogen 1.

In the present study, a low reciprocal agglutinin titre was found in pooled normal and acute phase mouse sera, both of which had a titre of 8. This agglutinin had no SLIN passive protective activity. When this was investigated further, 19 out of 20 individual sera samples contained this agglutinin with reciprocal titres ranging from 2-32. This agglutinin in pooled normal and acute phase sera was X mode B. pertussis specific and had not previously been reported. However this might be due to undilution of normal mouse sera not receiving attention. Cooper (1952) using a 1/5 dilution of individual normal mouse sera did not find agglutinins to formalized B. pertussis nor did Andersen (1953b) using a 1/10 dilution of pooled serum. Possibly these authors started their titrations at 1/5 and 1/10 dilution to avoid the normal agglutinin. This agglutinin in normal serum is unlikely to be due to previous priming of animals with B. pertussis as a booster effect would have occurred by day 15 post-institution, in acute phase sera and this was not seen. Possible intercurrent Bordetella bronchiseptica infection is also considered unlikely since no pathological changes were evident in normal mouse lungs. At no time were B. pertussis or other bacteria ever isolated on B.G. plated with lungs from normal mice.

The frequent absence of agglutinin development after a pertussis infection has been noted (Dolby and Stephens, 1973; Macaulay, 1979; Aftandelians and Connór, 1973) and also in the murine pulmonary infection (Andersen, 1953b). However where agglutinins did develop in

the last-mentioned article it was around day 35 and 42 post-instillation and the maximum reciprocal titre recorded in pooled serum was 80. Development of B. pertussis 18-323 and 18-334 X mode and 18-334 C mode agglutinins, in this study, reached respective peaks of 64, 128 and 8 agglutinating units by day 40-49 of a murine pulmonary infection, with a slight decrease to 32 in X mode titre and disappearance of C mode agglutinin on day 61. The more rapid disappearance of C mode agglutinins compared to the X mode specific agglutinins suggests that a different immunoglobulin class may be involved in the agglutination of C mode organisms.

The U.S. Standard Antipertussis Serum again showed a more marked immunological response than pooled convalescent mouse sera with a reciprocal titre of 16,000 to both X mode B. pertussis strains 18-323 and 18-334, and a reciprocal titre of 512 to C mode B. pertussis strain 18-334. The presence of C mode agglutinins in both the U.S. Standard Antipertussis serum and pooled day 40-49 mouse convalescent sera are obviously directed toward surface antigens not involved in the virulence of the organism but are common to X and C mode B. pertussis.

No agglutinin development was detected in pooled TBW at any stage of the infection. The absence of this immune response from the secretory surfaces is peculiar since it is thought to be involved in the clearance of organisms from the lung (Olson, 1975). However the inability to detect this immune response need not indicate that it does not exist but might be explained by the high dilution effect when sampling TBW.

The decrease in agglutinins to X mode B. pertussis on day 61

as well as the apparent absence in agglutinins in convalescent TBW prevented the correlation of reciprocal agglutination titre with SLIN passive protective activity. However absorption of 1 ml of pooled convalescent day 48 serum with 0.5 ml of 100 o.u. Phase I B. pertussis removed both the SLIN/mix passive protective activity and agglutinating activity. This together with the fact that U.S. Standard Antipertussis serum had correspondingly high SLIN/mix passive protective activity and reciprocal agglutination titre suggests there is a relationship between these two activities.

Immunoprecipitation as another means of serological identification of human pertussis has also been investigated, and precipitins to B. pertussis sonicates developed in 86% of paired sera from bacteriologically confirmed cases of pertussis (Aftandelians and Connor, 1973). Some sera sampled during the acute phase of infection had multiple bands which intensified then diminished during convalescence. Until the present study, there had been no report of development of precipitins during a murine infection; however this study showed that pooled convalescent sera but not pooled TBW did contain precipitins to B. pertussis sonicates. The U.S. Standard Antipertussis serum produced at least 3 lines compared to only 1 faint line in pooled convalescent sera.

By analysis of these pooled sera and TBW by qualitative immunoelectrophoresis, a more detailed precipitin profile was obtained. Pooled normal serum, as with the agglutination tests, did give a positive reaction in that one precipitin line was observed. However by the convalescence stage, 4 precipitin lines to B. pertussis sonicates

had developed. Pooled normal TBW did not contain any response to these sonicates but by day 15 one line had developed and by day 49 post-instillation 2 lines were present.

The U.S. Standard Antipertussis serum presented a much more complex pattern and obviously contained a variety of antibody specificities.

It would be interesting to find out towards which components of B. pertussis these immune responses were directed since the immunological response in pooled convalescent TBW, which gave two lines, might be directed against different components than those 4 immune responses found in pooled convalescent serum.

This would be evidence as to which immune responses are necessary in the secretory surfaces to prevent infection becoming established, eg anti LPS, and similarly which are important in the serum to prevent pharmacological changes associated with pertussis, eg anti pertussigen.

2.3 Immune responses towards individual virulence factors

Although it is not yet possible to say which of the B. pertussis virulence factor(s) is involved in producing the SLIN passive protective activity or the precipitin lines in qualitative immunoelectrophoresis, it was possible to investigate the immune responses to these active components of B. pertussis. These will be discussed in the sequence in which the components are thought to be involved in pathogenesis with the exception of the agglutinogens which have already been discussed.

Attachment of B. pertussis to the ciliated epithelium is thought to be due to F-HA. There have been conflicting reports on anti-HA development during the human infection with Winter (1953) finding only 7-14% of convalescent children with this antibody but Dolby and Stephens (1973) found all sera from infected children had anti-HA.

B. pertussis has two components with haemagglutinating activities: the LPF-HA (pertussigen) and the F-HA, with the purified form of the latter being 7 fold more active. As yet the development of an immune response to purified F-HA in the human or murine pulmonary infection has not been reported. However purified F-HA produced active protection in mice (Munoz et al, 1981) and i.p. anti-F-HA was passively protective in mice by the SLIN infection route (Sato et al, 1981).

In this investigation pooled sera from normal and B. pertussis mice contained antibodies to purified F-HA with the former having a reciprocal titre of 2 and the latter having a reciprocal titre of 32 on day 34 but returning to a reciprocal titre of 8 by day 49 post-instillation. Since anti-F-HA decreases in late convalescence as SLIN passive protective activity is increasing, there is no correlation between the two activities at this stage. However this does not mean that anti-F-HA is not involved in SLIN passive protective activity in pooled sera taken from early convalescence.

As with the agglutination test, the low anti-F-HA reciprocal titre in pooled normal sera was most probably not due to previous experience of B. pertussis and B. bronchiseptica, since no booster effect was detected in pooled day 15 acute phase serum. This antibody did not give rise to any SLIN passive protective activity.

The proposed role of F-HA, as stated previously, in pathogenesis is one of attachment of B. pertussis to the ciliated epithelium of the respiratory tract since Sato (1981) found anti-F-HA prevented attachment to such epithelium. Sato et al (1979) also demonstrated anti-F-HA bound to the pili-like appendages. This was not confirmed by Ashworth et al (1982) who in fact showed agglutinin 2 bound specifically to the appendages and not anti-F-HA. The involvement of agglutinin 2 in attachment has yet to be determined.

The disappearance of anti-F-HA agrees with the hypothesis by Pittman (1979) who proposed that F-HA is responsible for a short-term immunity which moreover would be needed at the secretory surfaces. However pooled TBW never developed detectable anti-F-HA which again, as with agglutinin development, may be explained by the high dilution of the secretions when washing out the lungs. Also non-development does not mean it would not be protective if it had developed.

After B. pertussis has attached to the ciliated epithelium, the organism multiplies and would be expected to synthesise the toxic components: HLT, LPS and pertussigen. The lack of anti-HLT in convalescent children's sera has been demonstrated (Anderson and North, 1943; Evans and Maitland, 1939) and has raised questions about its role in pathogenesis of the disease. However anti-HLT was passively protective in a murine pulmonary infection if the antiserum was administered as a mixture with challenge. In this investigation the anti-HLT response in pooled normal and infected sera and TBW was insufficient to neutralise a standard haemorrhagic dose of HLT. The absence of anti-HLT in convalescent TBW seems peculiar if this component plays an important role in virulence except that its low

immunogenicity may be responsible.

Turning now to the anti-LPS response, Ackers and Dolby (1972) found when determining the bactericidal activity of children's convalescent sera, an assay which is dependent on anti-LPS and complement, the bactericidal activity was variable. The latter assay cannot be compared to the one used in this study where no anti-LPS activity was found in pooled normal or infected mouse serum since the assay used here did not have an added source of bactericidal complement. Pooled day 40 convalescent TBW had an anti-LPS reciprocal titre of 64 by haemagglutination of LPS coated erythrocytes which diminished to a reciprocal titre of 4 by day 61. This response might play an important role in opsonization of B. pertussis thereby aiding phagocytosis and therefore clearance of organisms. Since anti-LPS decreased in late convalescence, the response does not correlate with SLIN passive protective development. However it could be responsible for SLIN/mix activity in early convalescence.

The magnitude of the anti-LPS response in convalescent TBW is doubly surprising since it was not detected in convalescent sera considering how dilute pooled TBW is in respect to pooled sera. It was therefore a local immune response.

The last toxin to be discussed is pertussigen (Munoz ^{and Bergman,} 1979) ^{et al,} referred to as LPF-HA by Sato ⁽¹⁹⁸¹⁾, has been proposed as the IC mouse protective antigen (Munoz ^{and Bergman,} 1979) and also as an SLIN protective antigen (U.S. Workshop, 1982). Accumulated evidence (Parker and Morse, 1973; Ortey, 1977) indicates that pertussigen alters the responsiveness of different cells to adrenergic and other agents, and interferes with cAMP metabolism. The proposed molecular mechanisms

by which pertussigen achieves these changes are by increasing activation of adenylate cyclase (Katada and Ui, 1982a, b; Katada et al, 1982.).

Pittman (1979), who referred to the component as Pertussis Toxin, proposed this antigen was responsible for the longer-lasting immunity associated with pertussis vaccines in the child. However development of an immune response to this component during either the human or the murine pulmonary infection has not previously been investigated. In this study 0.1 ml of pooled normal and infected sera and TBW were not able to neutralise 1 or 2 HSD₅₀ of pertussis vaccine. However since this assay might have been insufficiently sensitive, convalescent mice themselves were examined for their ability to neutralise both LPF and HSF in pertussis vaccine.

No neutralisation of HSF or LPF in 4 or 1.33 o.u. ml of pertussis vaccine occurred in day 49 convalescent mice. However day 63 convalescent mice were able to neutralise both activities in 1.33 and 0.44 o.u. ml (the HSD₅₀ was usually around 0.44 o.u. ml) of pertussis vaccine although the neutralisation of histamine sensitizing activity in 1.33 o.u. ml was less demonstrable than leucocytosis promoting activity. This might be related to the sensitivity of the assay, ie less pertussigen is required to stimulate histamine sensitivity than is required to promote leucocytosis.

None of the immune responses to the individual components correlate with the development of SLIN passive protective activity either in convalescent sera or TBW. This could well mean that SLIN passive protective activity in convalescent serum or TBW might be the resultant of several independent antibody specificities and actually reflects a combination of immune responses.

2.4 Immunity in secretions and sera

The non-specific inflammatory response has previously been measured by recording lung weight as an index of lung oedema (Andersen, 1953a). In this author's work the response peaked around day 14 post-instillation of challenge but was still maximum in some mice on day 21 (last recording day). In the present investigation lysozyme, as an index of leucocyte infiltration, was assayed in pooled TBW and in serum for comparison. This enzyme is contained in polymorphs and macrophages and is released into secretions when these cells lyse. Its peak concentration coincided with infection peak, day 15 post-instillation, and had returned to normal by day 63 when few, if any, organisms remained in the lungs. Not surprisingly this non-specific response was found only in pooled TBW and not in pooled sera.

2.5 Ig Class

Identification of the Ig class responsible for immunological responses in children has been limited to serum agglutination tests where the 19S and 9S Ig fractions were equally active (Dolby and Stephens, 1973). In this study IgG removal reduced agglutinin titres in day 61 convalescent serum. Murine B. pertussis specific Ig response to a sublethal pulmonary infection has been examined in both pooled serum and TBW (Geller and Pittman, 1973). Serum IgA started to increase on day 30 of infection and Pittman (1976) demonstrated parallelism between the time course of development of this murine serum IgA response and the development of LIN passive protective activity of sera from pertussis cases. In this investigation the

preliminary evidence obtained was that antibodies belonging to the IgG, IgA and IgE classes might all be involved in the SLIN passive protective activity of pooled day 61 convalescent serum of these, the IgG seemed to be about twice as active as the other two classes. The lack of IgM involvement was not surprising since after primary stimulation with other antigens IgM usually disappears after 30 days (Bauer et al, 1963). An additional method of showing serum IgE involvement in SLIN passive protection made use of the ability of this homocytotrophic antibody to remain bound to tissue for up to 48 hours. North (1946) demonstrated the ability of SLIN passively protective sera to remain active if given up to 48 h before a murine challenge. This result was verified for pooled day 61 convalescent serum in this study. Serum IgE elevation in other secretory surface diseases, ie gonorrhoea and syphilis have been reported (Green et al, 1976).

SECTION III FURTHER PERSPECTIVES

The direction of subsequent investigations could profitably be towards those areas where hitherto there has been a limitation in either suitable equipment or purified preparations from B. pertussis.

The murine sublethal pulmonary infection, as a model, falls short in its initiation of human pertussis since the typical characteristic cough of the latter infection does not occur. However perhaps adequate physiological instruments would permit detection of changes in murine respiratory function associated with the infection. The appearance of the mouse lungs at autopsy make it

difficult to believe that a normal physiological profile would be present since they are so heavily consolidated during the infection.

Immunological investigations should be in the following areas. Firstly the cell-mediated immune response has received little attention (Dolby and Stephens, 1973) although Cheers and Gray (1969) found intracellular survival of B. pertussis even with enhanced intracellular killing ability during a murine sublethal pulmonary infection.

Secondly, the nature of the mouse immune response to purified B. pertussis components would be more accurately described if detected by the sensitive enzyme linked immunoabsorbent assay. Once these purified components, such as F-HA, pertussigen etc, become freely available, monoclonal antibodies could be prepared and would reveal which Ig class(es) are involved in the immune response to each component. Also the monoclonal antibodies could be used in passive protection tests to determine which antibodies give protection and therefore which antigens are protective. The important protective antigen combinations in mice, would then provide a rational choice of protective components for investigation in man.

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APPENDICES

Appendix 1

1. Preparation of Media

1.1 1% CAA solution

Materials	grams
CAA (Difco technical)	10.0
MgCl ₂ ·6H ₂ O	0.1
CaCl ₂	0.016
NaCl	5.0

The above ingredients were dissolved in 1 l of distilled water and the pH adjusted to 7.6 with 1N. NaOH. The CAA solution was then autoclaved.

1.2 B.G. plates

The BG agar (10 g) was dissolved in 250 ml 4% (v/v) glycerol, autoclaved at 121°C for 15 min and allowed to cool to 45°C. The defibrinated horse blood (50 ml) previously heated to 37°C was added aseptically, mixed gently with agar base and poured in either 20 ml volume onto 90 mm or 10 ml volume onto 45 mm triple vent plastic petri dishes. Bubbles were removed by flaming and plates were stored in plastic bags and used within two weeks.

1.3 Modified Cohen and Wheeler Medium (Sato et al, 1973)

Materials	grams
Casamino Acids (Difco technical)	50
CaCl ₂ (anhydrous)	0.05

Materials	grams
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.0038
cysteine	0.15
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ *	0.05
Glutamic Acid	1
Glutathione *	0.05
KH_2PO_4	2.5
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	2
Nicotinamide (BDH, Poole, England)	0.15
NaCl	12.5
Soluble starch (BDH, Poole, England)	7.5
Tris (hydroxymethyl) aminomethane	30.4

All the components of the media except those labelled * were dissolved in 4.5 l of distilled water and the pH adjusted to 7.2. The components labelled * were dissolved in 100 ml of water, filter sterilized (Millipore) and then added just before use. The starch was dissolved in boiling water before adding it to the bulk of the medium. The volume was then made up to 5 litres and sterilized.

1.4 Modified Hornibrook medium

This medium is a modification of the recipe of Hornibrook (1940) as described by Parton and Wardlaw (1975) and consists of the following ingredients

Materials	per 10 l
(i) Casamino Acids (Difco Technical)	100 g
(ii) CaCl_2 (anhydrous)	0.02 g

Materials	per 10 l
(iii) NaCl	50 g
(iv) K ₂ Cl	2 g
Nicotinamide (BDH, Poole, England)	
(v) 0.1% (w/v) solution	10 ml
(vi) MgCl ₂ ·6H ₂ O, 1% (w/v) solution	25 ml
(vii) K ₂ HPO ₄ , 10% (w/v) solution	25 ml
(viii) Soluble Starch (BDH, Poole, England)	10 g
(ix) Glutathione (Sigma Chemical Company, St. Louis, Mo, USA) 0.1% (w/v) solution	100 ml

The ingredients (i)-(iv) were dissolved in 800 ml of distilled water and the freshly prepared solutions v, vi and vii added. The starch was suspended in 25 ml of distilled water and this added, with immediate mixing to 175 ml of boiling distilled water. This mixture was brought back to the boil and added to the rest of the medium. The volume was made up to 1 l and the pH adjusted to 7.0 with approximately 0.45 g of Na₂CO₃. This 10 times concentrate was dispensed in 100 ml amounts in screw cap bottles, sterilized by autoclaving at 121°C (15 psi) for 15 min, cooled and stored at 4°C.

Prior to use 100 ml of concentrate was added to 890 ml of distilled water in a 2 l dimpled conical flask and autoclaved at 121°C (15 psi) for 15 min. Immediately before use 10 ml of Seitz filter sterilized glutathione solution (0.1% (w/v)) was aseptically added to give Hornibrook X-medium.

Appendix 2

Protein determinations of tracheobronchial washings.

Reagent A

2% (w/v) Na_2CO_3 in distilled water

Reagent B

1% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in distilled water

2% (w/v) aqueous solution of sodium potassium tartrate.

Equal volumes of the above two solutions were mixed and the precipitate allowed to settle before using the supernatant.

Reagent C

To 50 ml of reagent A add 2 ml of reagent B. This reagent was prepared immediately before use and discarded after one day.

Reagent D

Folin and Ciocalteu phenol reagent (BDH, Poole, England).

Reagent E

Protein Standard 1 mg/ml^{-1} bovine serum albumin (Sigma Chemical Co., St. Louis, U.S.A.).

Appendix 3

Preparation of lysoplates

One gram of agarose (BDH Ltd., Poole, England) and 1% NaCl were dispensed into a bottle containing 90 ml of 0.06M sodium phosphate buffer (pH 6.4) (Appendix 4). The pH was re-adjusted to 6.4 with 40% (w/v) sodium hydroxide where necessary. The bottle was then autoclaved (121°C/15 min) cooled to 60°C and 60 mg freeze-dried M. luteus suspended in 10 ml buffer by treatment with ultrasound for 15 sec, added to each bottle with mixing. Aliquots of 5 ml were dispensed into 5 cm diameter petri dishes. When the agarose had set 3.4 mm diameter wells were cut in each with a No. 1 cork borer (Gallenkamp Ltd., London) and the plugs removed by suction.

Appendix 4Preparation of barbitone buffer pH 8.3

In approximately 450 ml distilled water 8.5 g barbitone sodium (BDH, Poole, England) was dissolved. The pH was adjusted to 8.3 using 1N HCl and made up to 500 ml with distilled water.

Ionagar

Ionagar (0.75 g) (Oxoid L28, Oxoid Ltd., Basingstoke, Hants) was dissolved in barbitone buffer.

Stain

This was a saturated solution of Amino Black (E. Gurr Ltd., Michrome Ltd.) in 2% Acetic Acid.

Appendix 5Preparation of sensitized Horse Red Blood Cells (H.R.B.C.)

Defibrinated horse blood was centrifuged, the R.B.C. washed three times in saline by centrifugation, and a 2.5% suspension was made in PBS buffer. The H.R.B.C. suspension was mixed with an equal volume of 0.1 mg/ml LPS (provided by Dr. A. Robinson, Centre for Applied Microbiology Research, Porton) which had been previously activated (Neter, 1950) by incubation for 18 h at 37°C in 0.01N NaOH in saline, and adjusted to pH 7 with dilute HCl. The mixture was incubated 1 h at 37°C, centrifuged, and washed 3 times in PBS buffer. The sensitized erythrocytes were resuspended in PBS buffer to make a 2% suspension for use in haemagglutination tests.

Appendix 6 Growth of B. pertussis and development of pathological changes in the lungs of intranasally infected mice : individual mouse data (summarized in Fig 3).

Experiment 1 (Infecting dose 4.0×10^5 cfu/mouse)				Experiment 2 (Infecting dose 1.0×10^5 cfu/mouse)			
Day post-infection	Mouse No.	cfu per lung mass	Pathology score	Day post-infection	Mouse No.	cfu per lung mass	Pathology score
4	1	1.8×10^6	1	5	1	1.1×10^7	1
	2	3.7×10^6	3		2	1.3×10^6	2
	3	5.1×10^6	1		3	5.5×10^5	0
					4	9.7×10^5	3
7	1	contamination	2	11	1	6.2×10^7	2
	2		1		2	3.7×10^7	3
	3		3		3	1.1×10^7	2
	4		3		4	contamination	2
11	1	1.6×10^7	4	13	1	contamination	1
	2	6.7×10^7	2		2	8.1×10^7	4
	3	8.3×10^7	3		3	3.2×10^7	2
	4	7.0×10^7	2		4	4.2×10^7	2
14	1	2.0×10^6	4	18	1	3.2×10^7	2
	2	9.1×10^6	3		2	1.0×10^6	3
	3	2.2×10^8	4		3	7.0×10^6	3
	4	1.7×10^8	4		4	3.2×10^6	2
21	1	8.3×10^4	2	24	1	$< 10^2$	3
	2	confluent	4		2	1.3×10^8	3
	3	$< 10^2$	4		3	$< 10^2$	2
		$< 10^2$	4		4	$< 10^2$	1
35	1	10^2	2	31	1	4.5×10^4	2
	2	9.8×10^5	3		2	2.5×10^3	2
	3	3.4×10^4	4		3	7.5×10^3	2
	4	$< 10^2$	4		4	$< 10^2$	2

Appendix 7 Mann-Whitney U-test on values in Table 14a for the calculation of significance of each group compared to the challenge alone

Type of Mouse ^a and (Day of Sampling)	Pooled sample	Values of U_1 and U_2 as calculated from the data and (critical value for $P=0.05$) ^b			
		Unheated Fluid		Heated 30 min at 56°C	
		Pathology	Culture	Pathology	Culture
N	TBW	44.5; 19.5 (13)	39; 9 (8)	-	-
I (49)	TBW	6 ; 58 (13)	0; 48 (8)	7; 41 (8)	7; 39 (8)
N	Sera	26 ; 38 (13)	16; 20 (6)	48; 16 (13)	21; 13 (6)
I (49)	Sera	5 ; 59 (13)	0; 48 (6)	5.5; 58.5 (13)	1; 29 (3)

a N = Normal; I = Infected

b For significance in the Mann-Whitney U-test, the smaller of the two experimental U-values must be equal to or less than the critical value of U for $P = 0.05$.

Appendix 8a : Pathology data for sera in Fig 12

Day post- instillation when sera obtained	Type of Mouse	Expt No	Challenge dose of bacteria in mix test (x10 ⁵)	No of mice with 2+ pathology					PD ₅₀ ml ⁻¹ (\pm 95% CL)
				No of mice challenged					
				1/2	1/20	1/100	1/200	1/240	1/ α
-	N ^a	1	1.0	4/5	NT	NT	NT	4/5	< 20
-	N	2	3.8	8/8	NT	NT	NT	9/9	< 20
15	I	2	2.8	6/8	NT	NT	NT	7/8	< 20
34	I	4	0.5	NT	1/10	2/10	3/9	6/10	6666 (86.9-33,333)
40	I	5	1.6	NT	2/10	7/10	7/10	NT ^b 8/9	1000 (190-6666)
42	I	1	0.4	1/10	1/10	4/9	7/10	NT 10/10	1818 (200 10,000)
47	I	3	1.5	NT	1/8	2/8	5/8	NT 7/8	5000 (571, 28571)
48	I	3	ND	NT	1/10	1/10	1/10	5/10 9/10	10,000 (1538, 51282)
49	I	2	1.0	1/10	1/10	3/10	5/10	NT 9/10	5000 (740, 28571)
61	I	6	0.67	1/200	1/600	1/1200	1/1800	6/7	33,333 (5882, 200,000)
61	I	7	0.67	1/6	1/7	3/8	6/8	6/7	13,500 (400-80,000)
U.S. Standard Antipertussis Serum									
				1/20	1/200	1/2000	1/20,000	1/200,000	571,428
				0/10	0/13	3/20	9/20	8/11	17/20

Appendix 8b : Culture data for sera in Fig 12

Day post- instillation when sera obtained	Type of Mouse	Expt No	Challenge dose of bacteria in mix test (x10 ⁵)	No of mice with 24 culture					PD ₅₀ ml ⁻¹ (\pm 95% CL)
				No of uncontaminated cultures					
				1/2	1/20	1/100	1/200	1/300	1/ α
-	N ^a	1	1.0	1/3	NT	NT	NT	NT	3/5
-	N	2	2.8	3/4	NT	NT	NT	NT	6/6
15	I	2	2.8	3/8	NT	NT	NT	NT	5/8
34	I	4	0.5	NT	1/8	1/6	4/9	4/7	7/10
40	I	5	1.6	NT	0/8	5/7	7/8	NT	5/7
42	I	1	0.4	N.D. ^b					
47	I	5	1.5	NT	1/8	2/8	4/8	NT	5/7
48	I	3	ND	NT	0/8	3/10	0/8	3/9	8/10
49	I	2	1.0	0/10	0/9	0/6	3/4	NT	5/9
61	I	6	0.67	1/200	1/600	1/1200	1/1800		4/6
				0/6	0/6	1/5	4/5		
61	I	6	0.67	0/8	0/8	4/8	5/6		4/6
U.S. Standard Antipertussis Serum									
				1/20	1/200	1/2000	1/20000	1/200000	1/ α
				0/4	NT	3/20	4/11	9/11	13/14
									444,444

a N = Normal ; I = Infected

b N.D. = Not defined

Appendix 8c : Pathology date for TBW in Fig 12

Day post- instillation when TBW obtained	Type of Mouse	Expt No	Challenge dose of bacteria in mix test (x10 ⁵)	No of mice with					2+ pathology	PD ₅₀ ml ⁻¹ (\pm 95% CL)
				No challenged						
				1/2	1/10	1/20	1/100	1/200	1/ α	
-	N ^a	1	1.0	4/4	—	—	NT ^b	—	4/5	< 20
-	N	2	2.8	5/8	—	—	NT	—	7/8	< 20
15	I	2	2.8	6/8	—	—	NT	—	5/8	< 20
34	I	4	1.6	3/11	3/9	9/9	NT	NT	8/8	125 (45,454)
40	I	5	1.5	6/10	3/4	4/4	NT	NT	8/8	32 (6, 133)
42	I	1	1.0	0/5	—	—	ND ^b	—	4/5	222 (54,1000)
47	I	5	2.45	2/10	4/10	5/7	NT	NT	9/9	500 (91, 2000)
48	I	3	0.5	0/8	2/8	2/8	8/8	NT	6/8	125 (30, 500)
49	I	2	1.1	1/10	NT	9/10	10/10	9/10	9/10	222 (46, 1000)
61	I	6	2.2	1/8	3/8	6/8	6/8	NT	7/8	1000 (250, 5000)
61	I	7	1.16	0/8	0/8	4/8	5/8	4/6	7/7	

a N = Normal; I = Infected

b NT = Not tested; ND = Not defined

Appendix 8d : Culture data for TBW in Fig 12

Day post- instillation when TBW obtained	Type of Mouse	Expt No	Challenge dose of bacteria in mix test (x10 ⁵)	No of mice with No of uncontaminated cultures					PD ₅₀ ml ⁻¹ ([±] 95% CI)
				1/2	1/10	1/20	1/100	1/200	
-	N ^a	1	1.0	3/4	—	NT ^b	—	—	< 20
-	N	2	2.8	6/8	—	NT	—	—	< 20
15	I	2	2.8	6/8	—	NT	—	—	< 20
34	I	4	1.6	2/6	5/8	8/9	NT	NT	100 (17, 666)
40	I	5	1.5	2/6	4/7	4/4	NT	NT	117 (20, 666)
42	I	1	1.0	0/2	—	NT	—	—	3/5
47	I	5	2.5	2/10	5/10	4/8	NT	NT	6/6
48	I	3	0.5	0/6	2/6	2/2	6/6	NT	2/3
49	I	2	1.1	1/5	NT	5/5	3/3	ND	2/3
61	I	6	2.2	1/8	2/5	3/7	3/5	NT	4/5
61	I	7	1.16	0/8	1/8	1/8	4/8	3/5	4/4

a N = Normal; I = Infected

b NT = Not tested.

Appendix 9 : Individual lung pathology and culture score of results from Table 19.

Absorption treatment of sera ^c with <u>B.pertussis</u> (o.u.)	Passive protective activity (individual mouse score)					
	Expt 1			Expt 2		
	Pathology	Culture	Pathology	Pathology	Culture	Culture
Nil	- - - - - ^b	- - - - - *	- - - - - *	- - - - - *	- - - - - *	- - - - - *
	- - - - - 2 2	- - - - - 4	- - - - - 1 2	- - - - - 1 2	- - - - -	- - - - -
200 o.u. Phase I	1 2 3 4 4	- - - - - 2 3 (NS)	2 3 3 4 (NS)	2 3 3 4 (NS)	- - 4 4 (NS)	- - 4 4 (NS)
	4 4 4 4 4	3 4 4 4 4	4 4 4 4 4	4 4 4 4 4	4+4+4+4+	4+4+4+4+
1000 o.u. Phase I	3 4 4 4 4	2 3 3 3 4 (NS)	2 3 3 3 4 (NS)	2 3 3 3 4 (NS)	2 2 3 3 (NS)	2 2 3 3 (NS)
	4 4 4 4 4	4 4 4 4 4	4 4 4 4 4	4 4 4 4 4	4 4 4 4	4 4 4 4
200 o.u. Phase IV	- - - - - *	- - - - - *	- - - - - *	- - - - - *	- - - - - *	- - - - - *
	- - 1 1 3	- - - - - 1 4	- - - - - 2 3 3	- - - - - 2 3 3	- - - - - 3 4	- - - - - 3 4
1000 o.u. Phase IV	- - - - - *	- - - - - *	- - - - - *	- - - - - *	- - - - - *	- - - - - *
	- - - 2 2	- - - - - 4 4	- - - - - 3	- - - - - 3	- - - - - 4	- - - - - 4
Controls						
Infection alone	2 3 3 3 4	- 2 3 3 3	- 2 2 3	- 2 2 3	- - 2 2	- - 2 2
	4 4 4 4 4	4 4 4 4 4	3 3 4 4	3 3 4 4	3 4 4 4	3 4 4 4
Normal sera absorbed with 200 o.u. Phase I	1 2 3 3 3 (NS)	2 3 3 3 4	NT ^d	NT ^d	NT	NT
	4 4 4 4 4	4 4 4 4 4				

a Absorption with both phases of B. pertussis was done with 2 different doses, 200 o.u and 1000 o.u.

b The statistical significance between the difference from the infection alone group by the Mann-Whitney U-test with NS = Not significant and * = significant at P = 5% level.

c All pooled convalescent sera were done at 1/10 diln.

d NT = Not tested

Appendix 10. : Individual lung pathology and culture score of results from Table 20

Absorption treatment of sera with <i>B. pertussis</i> (o.u.)	Passive protective activity (individual mouse score)			
	<u>Expt 1</u>		<u>Expt 2</u>	
	Pathology	Culture	Pathology	Culture
Nil	- - - - -	- - - - -	- - - - -	- - - - -
	- - - - 1	- - - - 2	- - - 1 2	- - - 2 2
200 o.u. Phase I	1 1 2 2 2	- - 1 3 3	1 3 3 4 4	- 2 2 4 4
	2 3 4 4	4 4 4 4	4 4 4 4 4	4 4 4 4
1000 o.u. Phase I	- - 1 2 2	- - - 2 2	1 2 2 2 3	- 1 2 2
	2 2 3 4	2 4 4	3 3 4 4 4	3 4
200 o.u. Phase IV	- - - - -	- - - - -	- - - - -	- - - - -
	- - 1 1	- - - - -	- 1 1 2 3	- - 2 2 4
1000 o.u. Phase IV	- - - - -	- - - - -	- - - - -	- 1 4
	- 1 2	- - - - -	- - - 1 2	
Controls				
Infection alone	2 2 4 4 4 4	2 4 4 4 4 4	1 3 3 3 4 4 4 4	- 3 4 4 4
Normal sera absorbed with 200 o.u. Phase I	NT ^a	NT	2 3 3 4 4 4 4 4	- - 2 4 4 4 4 4

a NT = Not tested

Appendix 11 : Individual lung pathology and culture score of results from Table 21

Time of administration of sera in relation to challenge (dilution)	Passive protective activity (Individual score)					
	Expt 1 ^a		Expt 2		Expt 3	
	Pathology	Culture	Pathology	Culture	Pathology	Culture
48 h before (1/20)	0 0 0 0 1 *	- ^b	0 0 1 1 2 *	0 4 3 4 (NS)	0 0 1 1 *	0 0 0 0 *
24 h before (1/20)	0 0 0 1 1 *	-	NT ^d	NT	NT	NT
6 h before (1/20)	0 0 0 0 1 *	-	NT	NT	NT	NT
Mixture (1/20)	0 0 0 0 0 *	-	0 0 0 0 0 *	0 0 0 0 1 *	0 0 0 0 *	0 0 1 (NS)
Mixture (1/200)	0 0 0 0 1 *	-	0 0 0 1 2 *	0 0 0 0 4 *	0 1 1 2 *	4 4 4 4 NS
Controls						
Challenge alone	1 1 2 2 2 2 2 3 3 4	-	3 4 4 4 4	3 4 4 4 4	1 4 4 4 4 4 4 4	2 4 4 4 4
Ether 2 days before challenge	NT	NT	2 3 3 3 4 (NS)	4 4 4 4 4 (NS)	1 2 2 2 (NS) 2 3 4 4	0 0 2 4 (NS) 4 4 4 4
CAA 2 days before challenge	NT	NT	1 2 3 3 4 (NS)	3 4 4 4 4 (NS)	0 2 2 2 (NS) 3 3 4 4	1 1 2 4 (NS) 4 4 4 4
Normal sera 2 days before	NT	NT	1 2 2 2 4 (NS)	0 2 3 4 (NS)	0 1 2 2 2 3 4 4 (NS)	0 3 3 4 (NS) 4

^a cfu of challenge of Expt 1 = 7×10^4 ; Expt 2 = 1.1×10^5 ; Expt 3 = 1.0×10^5 .

^b Expt 1 had no culture results due to complete contamination

^c The statistical significance when compared to infection alone group by the Mann-Whitney U-test
NS = Not significant and * = significant at the 5% level.

^d NT = Not tested

Appendix 12 : Individual lung pathology and culture sera of results from Table 22

Time of administration of TBW in relation to challenge (dilution)	Passive protective activity (Individual score)			
	Expt 1 ^a		Expt 2 ^a	
	Pathology	Culture	Pathology	Culture
48 h before (1/2)	0 1 2 3 4 (NS) ^c	- ^b	0 0 1 1 2*	0 0 3*
24 h before (1/2)	0 0 0 1 1*	-	0 1 2 2 4*	0 1 4 4 (NS)
6 h before (1/2)	0 0 0 1 1*	-	NT	NT
Mixture (1/2)	0 0 0 0 0*	-	0 1 1 2 2*	1 3 4 (NS)
Mixture (1/20)	0 0 0 0 1*	-	1 2 3 4 4 (NS)	0 3 4 4 (NS)
Controls				
Challenge alone	1 1 2 2 2 2 2 3 3 4	-	3 4 4 4 4	3 4 4 4 4
Ether 2 days before challenge	NT ^d	NT	2 3 3 3 4 (NS)	4 4 4 4 4 (NS)
CAA 2 days before challenge	NT	NT	1 2 3 3 4 (NS)	3 4 4 4 4 (NS)

a cfu of challenge in Expt 1 = 7×10^4 ; Expt 2 = 1.1×10^5

b Expt 1 had no culture results due to complete contamination

c The statistical significance when compared to infection alone group by the Mann-Whitney U-test
NS = Not significant and * = significant at the 5% level

d NT = Not tested

Appendix 13 : Determination by immunodiffusion of optimal concentrations to precipitate out IgG from pooled day 61 convalescent sera using goat antimouse IgG

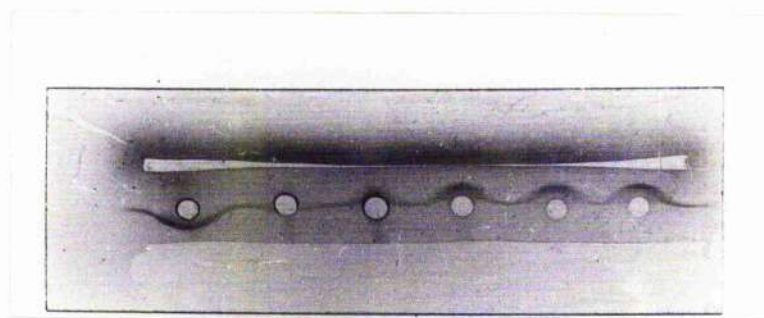
1	2	3	4	5	6	Anti IgG 1/10 ^a
0	0	0	0	0	0	Wells 1-6 ^b containing various absorbed sera
						C.S. 1/100 ^c

a Anti IgG = Goat antimouse IgG

b Wells 1-6 contained pooled day 61 convalescent sera at a 1/50 dilution which had been absorbed with the following dilutions of goat antimouse IgG:

Well 1	1/10	dilution of goat antimouse IgG				
Well 2	1/20	"	"	"	"	"
Well 3	1/40	"	"	"	"	"
Well 4	1/80	"	"	"	"	"
Well 5	1/160	"	"	"	"	"
Well 6	1/320	"	"	"	"	"

c CS = Pooled day 61 convalescent sera



Appendix 14 : Determination by immunodiffusion of optimal concentrations to precipitate out IgA from pooled day 61 convalescent sera using goat antimouse IgA

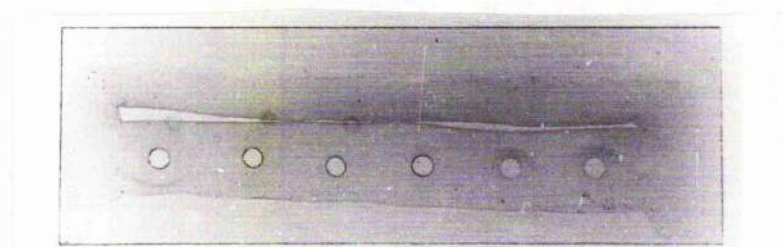
0	0	0	0	0	0	Anti IgA ^a 1/2 dilution
1	2	3	4	5	6	Wells 1-6 ^b containing various absorbed sera
						C.S. ^c 1/8 dilution

a Anti IgA = Goat antimouse IgA

b Wells 1-6 contained pooled day 61 convalescent sera at a 1/5 dilution which had been absorbed with the following dilution of Goat antimouse IgA

Well 1	1/2 dilution of Goat antimouse IgA					
Well 2	1/4	"	"	"	"	"
Well 3	1/8	"	"	"	"	"
Well 4	1/16	"	"	"	"	"
Well 5	1/32	"	"	"	"	"
Well 6	1/64	"	"	"	"	"

c C.S. = Pooled day 61 convalescent sera



Appendix 15 : Determination by immunodiffusion of optimal concentrations to precipitate out IgG from 10x concentrated pooled day 48 convalescent TBW using goat antimouse IgG

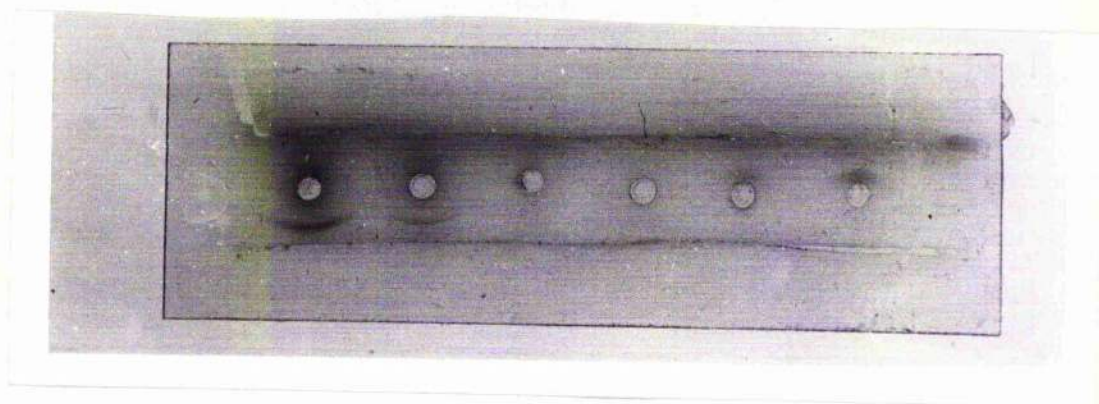
1	2	3	4	5	6	Anti IgG ^a 1/20
0	0	0	0	0	0	Wells 1-6 ^b containing various absorbed TBW
						C.TBW ^c 1/20

a Anti IgG = Goat antimouse IgG

b Wells 1-6 contained ^{10x} concentrated pooled day 48 convalescent TBW at a 1/10 dilution which had been absorbed with the following dilutions of Goat anti IgG

Well 1	1/2	dilution of Goat anti mouse IgG				
Well 2	1/4	"	"	"	"	"
Well 3	1/8	"	"	"	"	"
Well 4	1/16	"	"	"	"	"
Well 5	1/32	"	"	"	"	"
Well 6	1/64	"	"	"	"	"

c C.TBW = concentrated pooled day 48 convalescent TBW



Appendix 16 Determination by immunodiffusion of optional concentrations to precipitate out IgA from 10x concentrated pooled day 48 convalescent TBW using goat antimouse IgA

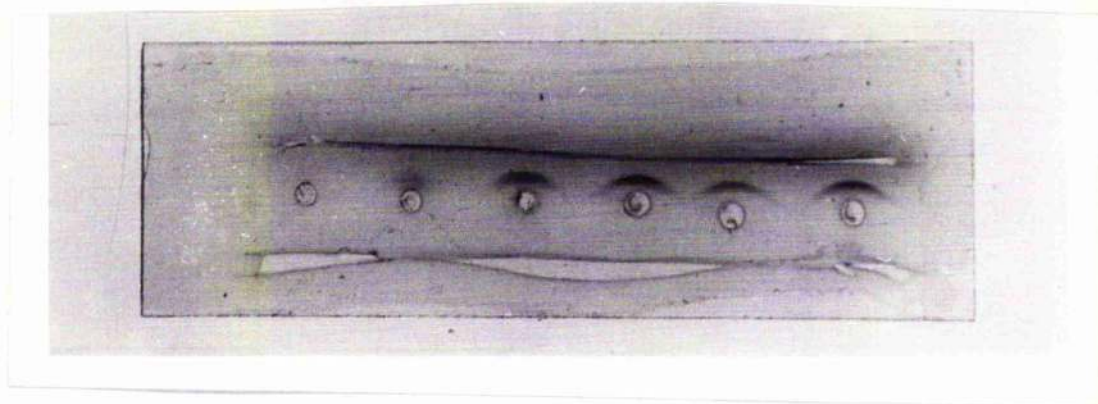
0	0	0	0	0	0	Anti IgA ^a 1/2 dilution
1	2	3	4	5	6	Wells 1-6 ^b containing various absorbed TBW
						C.TBW ^c Neat

a Anti IgA = Goat anti mouse IgA

b Wells 1-6 contained ^{10x} concentrated pooled day 48 convalescent TBW neat which had been absorbed with the following dilutions of Goat anti IgA

Well 1	Neat	Goat	anti	mouse	IgA	
Well 2	1/2	dilution	of	Goat	anti	mouse IgA
Well 3	1/4	"	"	"	"	"
Well 4	1/8	"	"	"	"	"
Well 5	1/16	"	"	"	"	"
Well 6	1/32	"	"	"	"	"

c C.TBW = concentrated pooled day 48 convalescent TBW



Appendix 17 : Individual experimental and cumulative totals for sera in Table 28

Treatment of sera	Dilution	Proportion with \geq 2 score			
		Passive protective activity (No infected or uncontaminated cultures)		Totals	
		Expt 1 ^a	Expt 2	Pathology	Culture
		Pathology	Culture ^c	Pathology	Culture
Untreated	1/100	1/5		1/5	1/5
	1/300	2/5		0/5	0/5
	1/600	3/5		5/5	5/5
IgM removed	1/150	1/5		0/5	0/5
	1/300	3/5		1/5	1/5
IgG removed	1/150	3/5		1/5	1/5
	1/300	4/5		3/5	3/5
IgA removed	1/150	3/5		1/5	1/5
	1/300	4/5		2/5	3/5
IgE removed	1/150	1/5		2/5	2/5
	1/300	1/5		2/5	2/5
Challenge alone		4/5		4/5	4/4

^a cfu of infecting dose in Expt 1 = 6×10^4 and Expt 2 = 1.1×10^5

^b The statistical significance of the difference when compared to the challenge alone group by the 2 x 2 contingency tables of Finney et al (1963)

NS = Not significant; * = significant at $P = 0.05$ and ** = significant at $P = 0.01$.

^c Complete contamination prevented lung culture results being recorded.

Appendix 18 : Individual experimental and cumulative totals for TBW in Table 29

Treatment of TBW	Dilution	Proportion with ≥ 2 score					
		Passive protective activity (No infected or uncontaminated cultures)		Expt 1 ^a		Expt 2	
		Pathology	Culture	Pathology	Culture	Pathology	Totals Culture
Untreated	1/5	0/5		0/5	1/5	0/10** ^b	1/5*
	1/25	1/5		4/5	2/5	5/10 (NS)	2/5 (NS)
	1/125	4/5		4/5	4/5	8/10 (NS)	4/5 (NS)
IgM removed	1/5	0/5		1/5	3/5	2/10*	3/5 (NS)
	1/25	1/5		4/5	3/5	5/10 (NS)	3/5 (NS)
IgG removed	1/5	3/5		5/5	4/5	8/10 (NS)	4/5 (NS)
	1/25	4/5		4/5	4/5	8/10 (NS)	4/5 (NS)
IgA removed	1/5	2/5		1/5	1/5	3/10*	1/5*
	1/25	3/5		NT	NT	3/5 (NS)	NT
IgE removed	1/5	3/5		NT	NT	3/5 NS	NT
	1/25	1/5		4/5	5/5	5/10 (NS)	5/5 (NS)
Challenge alone		5/5		5/5	5/5	10/10	5/5

a cfu of infecting in Expt 1 = 7×10^4 and Expt 2 = 2×10^5

b The statistical significance of the difference when compared to the challenge alone group by the 2 x 2 contingency tables of Finney et al (1963)

NS = Not significant; * = significant at $P = 0.05$ and ** = significant at 0.01 .